

ISOLATION AND STRUCTURAL DETERMINATION  
OF  
POLYPHENOLS IN TASMANIAN EUCALYPTS.

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by

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A thesis submitted in partial fulfilment  
of the requirements for the degree of

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APRIL, 1973.

Except as stated herein, this thesis contains no material which has been accepted for the award of any other degree or diploma in any university and, to the best of my knowledge, this thesis contains no copy nor paraphrase of material previously published or written, except when due reference is made in the text of the thesis.

*Richard B. Brown*

Signed:

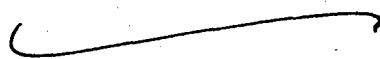


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## ABSTRACT.

Chapter 1 contains a brief review of chemotaxonomy as it relates to the classification of eucalypts.

Particular reference is made to a chromatographic survey by Hillis of the low molecular weight polyphenols on eucalypt leaves.

That survey provisionally identified a number of polyphenols of known structure but labelled other compounds "Unknowns", because of the lack of evidence for the identification of their structure.

The present work recounts the isolation and examination of the polyphenols in the leaves of three Eucalypt species important to Tasmania: E. delegatensis, E. sieberi and E. coccoifera.

Chapter 2 gives experimental details of the isolation of polyphenols from these species especially the isolation of some of Hillis' unknowns.

Chapter 3 describes the experimental techniques by which evidence for the identity of the isolated polyphenols has been gathered.

Chapter 4 then discusses the identity of these polyphenols.

First to be identified are a number of known polyphenols: gallic, gentisic and

protocatechuic acids; quercetin, myricetin, kaempferol; the flavonol glycosides, afzelin, rutin, quercitrin, isoquercitrin, hyperin and cannabiscitrin; ellagic acid and (+)-catechin.

Secondly, some of Hillis' unknowns have been isolated and identified.

Unknown D has been isolated

- (1) from E. sieberi and is identified as a mixture of flavanones: pinocembrin, alpinetin and a new natural product, 0,0-dimethylpinocembrin;
- (2) from E. coccifera and is identified as a mixture of flavones: apigenin and two C-methylflavones, sideroxylin and 4',5-dihydroxy-7-methoxy-6-methylflavone, the last identified provisionally.

Unknown A is identified as 4-O-methylellagic acid, a new ellagic acid ether.

Unknown B is provisionally identified as a new lignan, 1,5-dimethyl-2,6-bis (trihydroxyphenyl) furo-[1,5-c] furan.

Unknown F is tentatively given the structure of a tetrahydroxydibenzofuran-dicarboxylic acid isomer.

Throughout the discussion the use of chromatography of mixtures to provide evidence for chemotaxonomy is critically considered.

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### ACKNOWLEDGEMENTS.

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Dr. W.E. Hillis, Senior Principal Research Scientist, C.S.I.R.O., Division of Applied Chemistry, Officer-in-Charge, Wood and Forest Science Section, suggested the project, gave continual encouragement and advice and supplied many authentic samples; to him special thanks are given.

Dr. T.J. Batterham, John Curtin School of Medical Research, Australian National University, gave the use of his laboratory and facilities during a two-week stay in that laboratory, together with his interest and advice.

Finally, thanks are due to Mr. L. Brasch, Librarian, Associated Pulp and Paper Mills Ltd's Library, Burnie and to the Librarian, Forest Products Laboratory Library, Melbourne, for the use of the facilities of their respective libraries.

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POLYPHENOLS OF TASMANIAN EUCALYPTS.

CHAPTER 1.

INTRODUCTION.



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## CHAPTER 1.

### 1.0. EUCALYPT CLASSIFICATION AND CHEMOTAXONOMY.

The classification of eucalypt species in use today is Blakely's "A Key to the Eucalypts"<sup>16</sup> based on the so-called antheral system of Bentham. A second classification used by Blakely was based on general morphology but in some cases it contradicted the first.

While the original key has anomalies it remains the only complete system evolved and is the key upon which any classification used in this study is based.

The reliable classification of this difficult genus requires as many taxonomic criteria as possible.

In recent years increasing study has been made of wood anatomy,<sup>28,29</sup> bark anatomy,<sup>23</sup> cytology,<sup>101</sup> pollen grains,<sup>91</sup> seed coat anatomy,<sup>34</sup> and floral morphology<sup>20,21</sup> as taxonomic criteria.

Further it has been proposed that the chemical composition of various plant tissues could also be a useful criterion in taxonomy,<sup>48</sup> particularly as it expresses its results in different terms from those of botany and thus provides an independent check upon

classification. Bate-Smith,<sup>11</sup> Hasegawa,<sup>42</sup> Horn et al.<sup>59</sup> and more recently and extensively, Hillis,<sup>47</sup> have studied the chemical composition of a variety of plants and plant tissues.

Baker and Smith<sup>7</sup> first brought the focus of chemotaxonomy to bear on the Eucalypt genus in 1890 concentrating on the essential oil composition in eucalypt leaves.

Various plant tissues have been studied to decide which would provide the most reliable chemotaxonomic guide.

Wood and phloem extractives have proved of limited usefulness although heartwood extractives have shown more promise,<sup>43</sup> while the greatest attention has been given to leaf extractives which seem to provide a more reliable guide to phenotype than extractives from other plant tissues.

Different groups of compounds found in leaf tissue have been studied from the chemotaxonomic viewpoint.

In addition to the study of essential oils by Baker and Smith<sup>7</sup> and their successors, leaf waxes have been examined by Horn and his co-workers<sup>59</sup> who found them to be composed mainly of long-chain  $\beta$ -diketones with little promise of taxonomic significance.

#### 1.1. HILLIS' SURVEY OF EUCALYPT POLYPHENOLS.

Hillis<sup>47</sup> and others have suggested that compounds of low molecular weight in the leaves of eucalypts could provide a more useful taxonomic criterion.

Hillis' extensive survey of over 300 eucalypt species used the pattern of low molecular weight polyphenols in conjunction with other criteria to draw conclusions about eucalypt classification.

This examination was largely chromatographic, as far as possible using standard markers, and Hillis pointed out the need of extending the work by employing isolation and standard procedures of identification.<sup>48</sup> He proposed thereby to verify the identity of key polyphenols as well as to identify other polyphenols to which not even provisional identity was ascribed but which were simply labelled "Unknowns".

The present work takes the results of Hillis' survey as they apply to Tasmanian eucalypts, in particular to three species, and by isolation and techniques of structural analysis confirms the provisional identity of many of Hillis' polyphenols. It establishes the identity of some unknowns and isolates and identifies an additional polyphenol hitherto unrecorded by Hillis.

Further, the most abundant glycosides in each species are characterised to complete the chemotaxonomic picture.

As a result of this work a question is raised about the advisability of using chromatography alone as a guide even to provisional identity of compounds where overlying spots on the chromatogram are common. Especially is this true when some significance is attached

to such provisional identity in classification.

This is not to deny the validity of the use of chemical criteria in taxonomy in conjunction with other criteria but to suggest that the identity of compounds which are put forward as playing a significant role in classification must be accurately known if the conclusions based on their identity are to be reliable.

Two species of the Fraxinales or Ash series were examined for comparison of their polyphenolic content, E. delegatensis and E. sieberi. While not endemic both species have particular significance for Tasmania.

E. delegatensis (R.T. Baker)<sup>16a</sup> or E. gigantea (Hook f.)<sup>16a</sup> is particularly important to the State as a source of paper pulp and as a timber, Tasmanian Oak.

E. sieberi (L. Johnson)<sup>16a</sup> or Tasmanian Ironbark grows locally in the north-east from Coles Bay to Georges River between the coast and the headwaters of the South Esk River. Attempts to grow E. sieberi in large stands in other parts of the State for paper-making purposes have failed.

Although E. sieberi grows also in the sub-alpine regions of Victoria and southern New South Wales, Hillis found in his survey that the chemical composition of the Tasmanian samples was significantly different from that of the samples gathered on the mainland.<sup>49</sup>

E. coccifera (Hook f.)<sup>16b</sup> or Tasmanian Snow Gum grows in sub-alpine conditions at altitudes of 2000-4500

7  
feet. A Tasmanian endemic, it is widespread except in the north-east of the State.

Time permitted examination of only one of the Piperitales or Peppermint series, E. coccifera, although comparison of the polyphenol content of a second species of the series would have proved useful for chemotaxonomy.

#### 1.2. HILLIS' KNOWN POLYPHENOL AGLYCONES AND GLYCOSIDES.

Table 1 lists the aglycones chromatographically identified by Hillis together with those isolated and identified during the course of this current work. The flavonol glycosides isolated are listed in Table 2.

Other polyphenols, provisionally called caffeic, chlorogenic and sinapic acids and Hillis' unknown H, were observed in chromatograms in the course of this study but were not isolated, so they are not listed in Table 1.

The structures corresponding to Hillis' known aglycones listed in Table 1 are given in Chart 1, while the structures of the known aglycones and glycosides actually isolated here are given in Chart 2 on page 11.

As Charts (1) and (2) show, there is substantial agreement between the known polyphenols identified by Hillis and those isolated in this work. But one should note the isolation of a third hydroxybenzoic acid, protocatechuic acid, which is not recorded by Hillis throughout his survey, although it is known to be widely distributed among the higher plants.<sup>62</sup>

TABLE 1.

1. Polyphenol aglycones chromatographically identified by Hillis (H) and identified after isolation by Brown (B).

kaempferol	(H & B)	protocatechuic acid	(B)
quercetin	(H & B)	a catechin (H) = (+)-catechin	(B)
myricetin	(H & B)	chlorogenic acid	(H)
gallic acid	(H & B)	ellagic acid	(H & B)
gentisic acid	(H & B)		

2. Polyphenol aglycones called unknowns by Hillis and structurally identified by Brown.

<u>HILLIS</u>	<u>BROWN</u>	<u>SPECIES</u>
<u>unknown A</u>	4-O-methylellagic acid	<u>E. sieberi.</u>
<u>unknown B</u>	1,5-dimethyl-2,6-bis(3,4,5-trihydroxyphenyl) furo-[1,5-c] furan	<u>E. sieberi.</u>
<u>unknown D</u>	0,0-dimethylpinocembrin	<u>E. delegatensis.</u>
	pinocembrin )	
	)	
	alpinetin )	<u>E. sieberi.</u>
	)	
	0,0-dimethylpinocembrin )	
	apigenin )	
	)	
	sideroxylin (4',5-dihydroxy-7-methoxy-6,8-dimethylflavone))	<u>E. coccifera.</u>
	)	
	4',5-dihydroxy-7-methoxy-6-methylflavone )	
	)	
<u>unknown F</u>	3,4,7,8-tetrahydroxy-dibenzofuran-1,5-dicarboxylic acid.	<u>E. sieberi.</u>

TABLE 2.

Flavonol glycosides isolated and identified in this work.

kaempferol-3-rhamnoside (afzelin)

myricetin-3-glucoside (cannabiscitrin)

quercetin-3-glucoside (isoquercitrin)

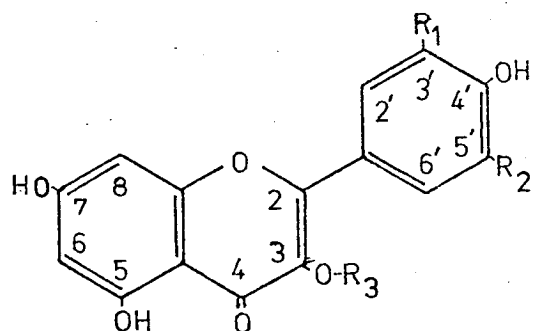
quercetin-3-rhamnoside (quercitrin)

quercetin-3-galactoside (hyperin)

quercetin-3-rhamnosylglucoside (rutin)

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$R_1, R_2 = H, R_3 = \text{Rhamnosyl}$ ; Afzelin

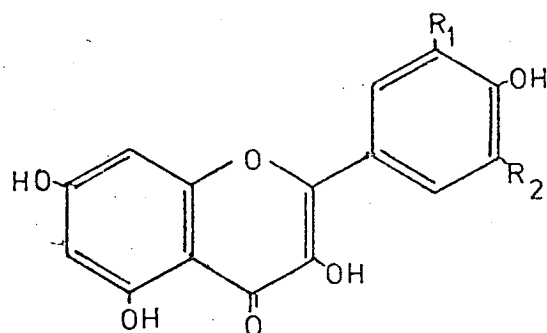
$R_1 = OH, R_2 = H, R_3 = \text{Rhamnosylglucosyl}$ ; Rutin

$R_3 = \text{Rhamnosyl}$ ; Quercitrin

$R_3 = \text{Glucosyl}$ ; Isoquercitrin

$R_3 = \text{Galactosyl}$ ; Hyperin

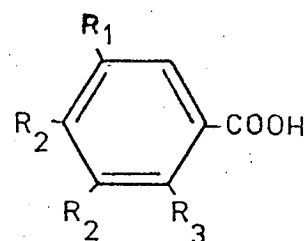
$R_1, R_2 = OH, R_3 = \text{Glucosyl}$ ; Cannabiscitrin



$R_1, R_2 = H$ ; Kaempferol

$R_1 = OH, R_2 = H$ ; Quercetin

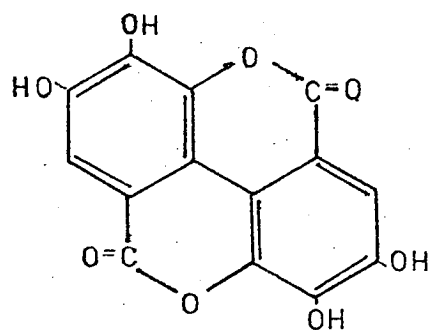
$R_1, R_2 = OH$ ; Myricetin



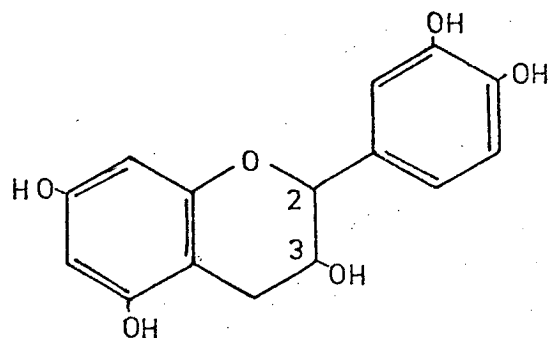
$R_1, R_2 = OH, R_3 = H$ ; Gallic acid

$R_1, R_3 = OH, R_2 = H$ ; Gentisic acid

$R_2 = OH, R_1, R_3 = H$ ; Protocatechuic acid

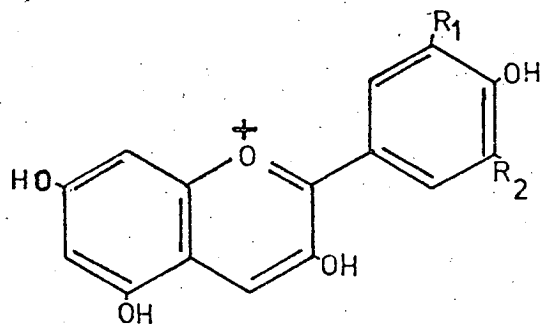


Ellagic acid



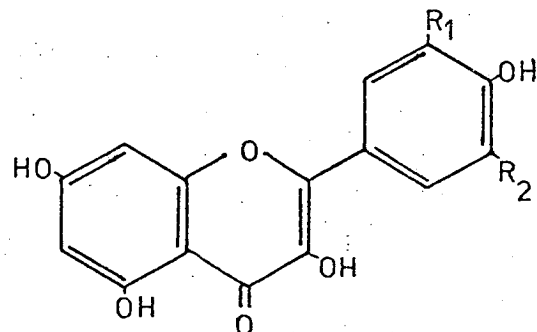
(+)-Catechin (2R:3S)

CHART 1. HILLIS' KNOWN POLYPHENOLS and GLYCOSIDES  
ISOLATED from TASMANIAN EUCALYPTS.



$R_1 = \text{OH}, R_2 = \text{H}$ ; Cyanidin

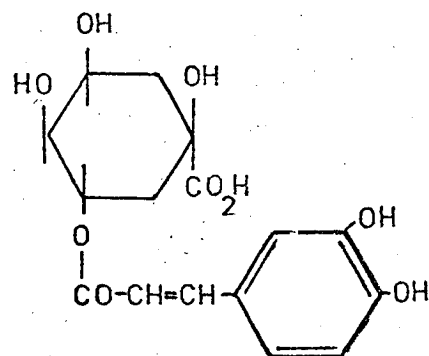
$R_1, R_2 = \text{OH}$ ; Delphinidin



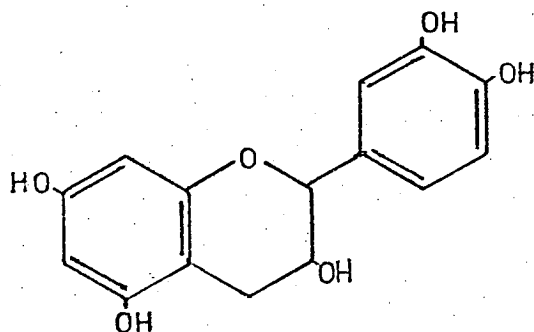
$R_1, R_2 = \text{H}$ ; Kaempferol

$R_1 = \text{OH}, R_2 = \text{H}$ ; Quercetin

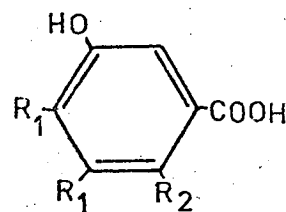
$R_1, R_2 = \text{OH}$ ; Myricetin



Chlorogenic acid



Catechin

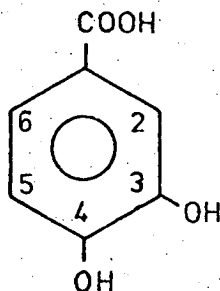


$R_1 = \text{OH}, R_2 = \text{H}$ ; Gallic acid

$R_2 = \text{OH}, R_1 = \text{H}$ ; Gentisic acid

CHART 2. HILLIS' MAIN PHENOLIC COMPONENTS IN SOLUTION AFTER  
ACID TREATMENT OF EUCALYPT LEAVES.

In addition to the identification of protocatechuic acid ( 1 ), a more detailed identification has been made of the catechin isolated.



( 1 )

### 1.3. HILLIS' UNKNOWN POLYPHENOLS.

The major concern of the present study has been the isolation and identification of unknown compounds in these species. The structures of the compounds isolated from the ashes with chromatographic behaviour similar to that of Hillis' unknown D are given in Chart 3 and are seen to be flavanones, one of which is a new compound. Whereas the compounds isolated from the peppermint and also called unknown D by Hillis are shown in the same chart to be flavones, of which two are rather rare C-methylflavones.

Hillis stresses the importance of unknown D in chemotaxonomy, states that it is found only in the Renantherae section of Blakely's classification<sup>49</sup> and

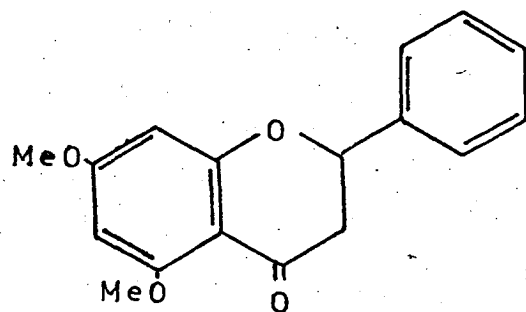
uses the compound to indicate association between numerous renantherous species.<sup>49</sup> He also states this unknown is present in the peppermint, E. coccifera, although he points out the anomalous chemical composition of this species.<sup>49</sup>

In proposing that unknown D is not a single component but a mixture and indeed a different mixture in the ashes and the peppermint examined, the present work raises doubts as to the taxonomic significance of unknown D.

Unknown A has been identified as a monomethyl ether of ellagic acid (Chart 3), previously unreported in the literature. This is in substantial agreement with the tentative identification made by Hillis who referred to it as an "ellagic acid-like" compound.<sup>48</sup> It was found in his survey that about half of the species examined contained the compound in varying amounts but the species were spread throughout the genus in such a random manner that the presence of this compound was apparently not significant to classification.

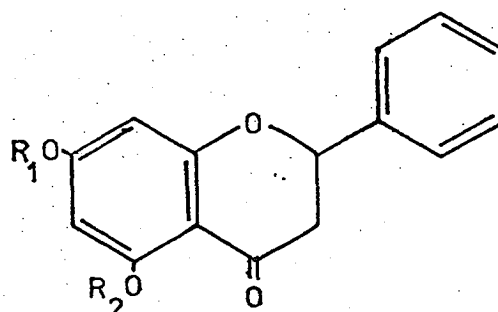
Unknown B was also described by Hillis as "ellagic acid-like" because of its chromatographic behaviour.<sup>48</sup> A compound from E. sieberi isolated and reported upon in this thesis has properties on paper chromatograms similar to those of unknown B, but on spectral evidence seems to be not a derivative of ellagic acid but a new lignan.

E. delegatensis



0,0-Dimethylpinocembrin

E. sieberi

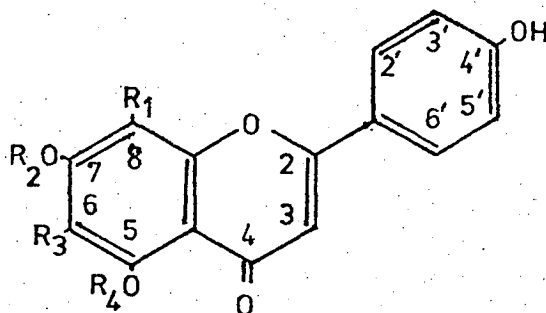


$R_1, R_2 = H$  ; Pinocembrin

$R_1 = H, R_2 = Me$  ; Alpinetin

$R_1 = R_2 = Me$  ; 0,0-Dimethylpinocembrin

E. coccifera

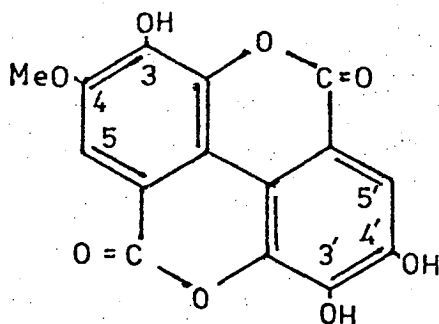


$R_1, R_2, R_3, R_4 = H$  ; Apigenin

$R_1, R_2, R_3 = Me, R_4 = H$  ; Sideroxylin

$R_2, R_3 = Me, R_1, R_4 = H$  ; 5,4'-Dihydroxy-7-methoxy-6-methylflavone

UNKNOWN A



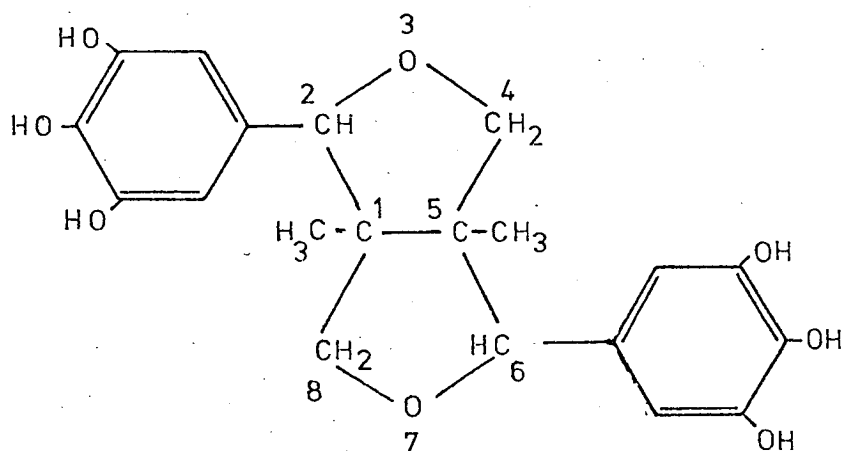
E. sieberi

4-O-Methylcinnamic acid

Finally, unknown F is tentatively identified now as a dibenzofuran shown in Chart 4. Hillis found that this compound was spread erratically throughout the Eucalypt genus and he based no chemotaxonomic conclusions upon its presence or absence.<sup>48</sup>

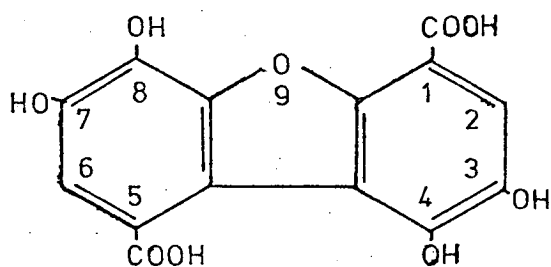
It was a particular difficulty in the isolation of this polyphenol that ellagic acid tended to remain as an impurity. Frequently unknown F spots on chromatograms showed not as an authentic yellow but as an ambiguous brown colour, highlighting the problem of provisional chromatographic identification. G.l.c. methods of trimethylsilyl ether separation<sup>44,45,99</sup> of this unknown were not attempted, as explained later.

---



### UNKNOWN B

1,5-dimethyl-2,6-bis(3,4,5-trihydroxyphenyl)furo[1,5-c]furan



### UNKNOWN F

3,4,7,8-tetrahydroxydibenzofuran-1,5-dicarboxylic acid

## CHAPTER 2.

### ISOLATION - EXPERIMENTAL.



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## CHAPTER 2.

### 2.0. ISOLATION - GENERAL PROCEDURES.

Since the isolation techniques of liquid-liquid extraction and chromatography followed for all three species were similar, these techniques are given first as general procedures.

Small departures from these general processes as well as additions to them for a particular species are then given separately as special procedures, (2.1, page 27 ).

#### 2.0.1. SOURCES OF LEAVES.

Samples of leaves of E. delegatensis examined were obtained from Associated Pulp and Paper Mills Ltd's forests at Meander. Leaves of E. sieberi were collected at Fingal and those of E. coccifera on Mt. Fenton, Mt. Field National Park, Hobart.

#### 2.0.2. LIQUID-LIQUID EXTRACTION.

Dried milled leaves of each eucalypt (2 kg) were exhaustively extracted with methanol and the extract, concentrated in vacuo to 2 l, was poured in a thin stream into 10 l of vigorously stirred water. The green waxy material which precipitated was filtered, re-dissolved in methanol and re-precipitated as before. The process was repeated until paper chromatograms showed the precipitate showed no polyphenols.

The combined aqueous methanolic extracts, concentrated in vacuo to 3 l, were extracted with

petroleum ether (40°-60°) until chromatograms showed that all chlorophylls, waxes and oils had been removed.

The aqueous methanolic concentrate was carried through a series of liquid-liquid extractions, summarised in Chart 5, to be read with the legend in Chart 6, pages 21,22.

### 2.0.3. CHROMATOGRAPHY.

Many of the chromatographic techniques used in this work have found application not only in the isolation but also the identification of polyphenols. The materials used are grouped here for convenience but their use is detailed in Chapter 2.1, Special Procedures.

References to this section will also be made in Chapter 3 which concerns identification of polyphenols.

Legend overleaf.

I or IH

(II) Ether extraction.

(III) Bicarbonate extraction.

(VII) Ethyl acetate extraction.

(VI)

(IV) Acidification plus ether extraction.

Not examined further.

(V)

(VIII) Bicarbonate extraction.

(XII) n-Butanol extraction.

(XI)

(IX) Acidification plus ethyl acetate extraction.

(XIII) Bicarbonate extraction.

Not examined further.

(X)

(XVII) Not examined further.

(XVI)

(XIV) Acidification plus butanol extraction

Not examined further.

(XV)

CHART 5. LIQUID-LIQUID EXTRACTION FLOW CHART.

- (I) An aqueous methanolic extract of eucalypt leaves was concentrated under vacuum and extracted with petroleum ether to remove chlorophylls, waxes and oils.
- (IH) Portion of the aqueous methanolic extract from (I) was hydrolysed with 2M hydrochloric acid.

#### ETHER EXTRACTION.

- (II) The extract from (I) or (IH) was extracted continuously with ether and the ether extract concentrated to 500 ml.
- (III) The ether concentrate from (II) was extracted with 5% aqueous sodium bicarbonate (6 x 100 ml. portions).
- (IV) The aqueous bicarbonate extract from (III) was acidified, re-extracted with ether and this ether extract concentrated, becoming (V).
- (VI) The ether extract from (III), i.e. after bicarbonate extraction, was concentrated.

#### ETHYL ACETATE EXTRACTION.

- (VII) The aqueous methanolic extract from (II), i.e. after ether extraction, was extracted continuously with ethyl acetate and the ethyl acetate extract concentrated.
- (VIII) The ethyl acetate extract from (VII) was extracted with 5% aqueous sodium bicarbonate.

#### CHART 6 LEGEND

- (IX) The aqueous bicarbonate extract from (VIII) was acidified, re-extracted with ethyl acetate and the ethyl acetate extract concentrated, becoming (X).
- (XI) The ethyl acetate extract from (VIII), i.e. after bicarbonate extraction was concentrated.

#### n-BUTANOL EXTRACTION.

- (XII) The aqueous methanolic extract from (VII) was hand extracted with n-butanol and the butanol extract concentrated.
- (XIII) The butanol extract from (XII) was extracted with 5% aqueous sodium bicarbonate.
- (XIV) The aqueous bicarbonate extract from (XIII) was acidified, re-extracted with n-butanol and the butanol extract concentrated, becoming (XV).
- (XVI) The butanol extract from (XIII), i.e. after bicarbonate extraction, was concentrated, becoming (XVI).
- (XVII) The aqueous methanolic extract from (XII), i.e. (XVII) was not examined further.

#### CHART 6. LEGEND TO ACCOMPANY FLOW CHART 5.

2.0.3.1. PAPER CHROMATOGRAPHY.

Paper chromatography was carried out on either Whatman No 1 or Whatman No 3MM paper using the following solvent systems:

<u>Solvent system.</u>	<u>Volume ratio.</u>	<u>Abbreviation adopted.</u>
1. <u>n</u> -butanol- acetic acid- water <sup>48</sup>	(6:1:2)	BAW (612)
2. <u>n</u> -butanol- acetic acid- water <sup>39</sup>	(4:1:5 upper phase)	BAW (415)
3. <u>t</u> -butanol- acetic acid- water <sup>75a</sup>	(3:1:1)	TBA
4. benzene- acetic acid- water <sup>54</sup>	(6:3:7)	BeAW
5. acetic acid- water <sup>48</sup>	(6:94)	6HOAc.
6. acetic acid- water <sup>75a</sup>	(15:85)	15HOAc.
7. hydrochloric acid- acetic acid- water <sup>54</sup>	(3:30:10)	Forestal = F
8. phenol- water <sup>39</sup>	(saturated, upper phase)	PhOH
9. ethyl acetate- formic acid- water <sup>57</sup>	(10:2:3)	EFW

Systems 1-9 were used in the development of either two-dimensional analytical chromatograms or one-dimensional preparative chromatograms of aglycones and glycosides.

<u>Solvent system.</u>	<u>Volume ratio.</u>	<u>Abbreviation adopted.</u>
10. ethyl acetate- pyridine- water <sup>10</sup>	(12:5:4)	EPW
11. <u>n</u> -butanol- pyridine- water <sup>10</sup>	(6:4:3)	BPW
12. acetone- water <sup>39</sup>	(80:20)	AcW

Systems 10-12 were used in the one-dimensional analytical chromatography of sugars.

The chromatograms were examined under u.v. light at wavelengths of 365 nm. and 254 nm. before and after exposure to concentrated ammonia vapour.

Polyphenol spots and bands on paper were visualised by spraying with:

<u>Spray.</u>	<u>Abbreviation.</u>	<u>Class of compound detected.</u>
1. ferric chloride (1%)- potassium ferricyanide (1%) <sup>10</sup>	FeFe	<u>all phenols</u>
2. diazotised <u>p</u> -nitroaniline (0.05%) in aqueous 20% sodium acetate <sup>103</sup>	pNA	<u>all phenols</u>
3. vanillin in conc. hydrochloric acid (1%) <sup>13</sup>		<u>flavan-3-ols</u>
4. ethanolic <u>p</u> -toluenesulphonic acid (3%) <sup>96</sup>		<u>flavan-3-ols</u>
5. sodium borohydride in isopropyl alcohol (1%) <sup>33</sup> followed by acid fuming		<u>flavanones</u>
6. diazotised sulphanilic acid <sup>85</sup>		<u>lignans</u>
7. chlorine vapour followed by 10% aqueous sodium sulphite <sup>80</sup>		<u>lignans</u>

Sugar spots were visualised with:

8. aniline hydrogen phthalate<sup>73</sup> (1% aqueous)
9. aniline phosphate<sup>3</sup> (1% aqueous)
10. p-anisidine hydrochloride in butanol (1%)<sup>17,75b</sup>  
followed by heat (130°)

#### 2.0.3.2. THIN LAYER CHROMATOGRAPHY.

Chromatoplates were prepared both in analytical (0.1 mm) and preparative thickness (0.3 mm) using the following adsorbents:

1. Camag silica, Kieselgel DSF-5, and Merck G.F.  
254 Kieselgel.
2. Camag Cellulosepulver DF.
3. Polyamide (Woelm) - silica- rice starch.<sup>82</sup>

Solvent systems used in thin layer chromatography were:

<u>Solvent system.</u>	<u>Volume ratio.</u>	<u>Abbreviation adopted.</u>
1. methanol- chloroform- petroleum ether (60°-80°) <sup>54</sup>	(2:4:7)	MCP
2. chloroform- acetic acid <sup>54</sup>	(6:1)	CA
3. chloroform- ethyl acetate- formic acid <sup>54</sup>	(5:4:1)	CEF
4. toluene- ethyl formate- formic acid <sup>54</sup>	(5:4:1)	TEF
5. benzene- pyridine- formic acid <sup>58</sup>	(36:9:5)	BPF



Spots and bands on chromatoplates were visualised by spraying with:

1. antimony (III) chloride (10% in  $\text{CHCl}_3$ ) followed by heating.<sup>58</sup>
2. diazotised p-nitroaniline (0.05%) in 20% aqueous sodium acetate.<sup>36</sup>
3. basic lead acetate (25% aqueous).<sup>58</sup>

#### 2.0.3.3. COLUMN CHROMATOGRAPHY.

The following adsorbents and eluting solvents have been used in one or other isolation:

<u>Adsorbent.</u>	<u>Eluent.</u>
1. Polyamide (Grisamid, Type TPU, Knapsack, Grisheim).	<u>ethanol-water</u>
2. Cellulose (Whatman).	<u>aqueous acetic acid</u>
3. Silica (Kieselgel) 200 mesh.	chloroform:ethyl acetate: <u>formic acid</u> (7:4:0:5, V:V:V)

#### 2.0.3.4. GEL FILTRATION.

The gel used as the stationary phase was Sephadex LH 20 with ethanol as organic eluent.

#### 2.0.3.5. GAS-LIQUID CHROMATOGRAPHY.

Gas-liquid chromatography of trimethylsilyl ether (TMS) derivatives of plant polyphenols showed g.l.c. to be a very useful addition to paper chromatography and t.l.c. in the analysis of flavonoids.<sup>44</sup>

Lack of suitable gas chromatographic resources frustrated further attempts to subfractionate some of Hillis' unknowns, particularly unknown F.

## 2.1. ISOLATION - SPECIAL PROCEDURES.

The following isolation methods for each species were followed in conjunction with, or as departures from, the General Procedures in Charts 5,6, pages 21,22.

### 2.1.1. LIQUID-LIQUID EXTRACTION.

#### 2.1.1.1. E. delegatensis.

##### Hydroxybenzoic acids, quercetin, ellagic acid.

The concentrated ether extract (VI) chromatographed two-dimensionally showed the presence of mainly hydroxybenzoic acids, quercetin and ellagic acid.

Upon further concentration of (VI) in vacuo impure ellagic acid precipitated which, after filtering, was crystallized from aqueous ethanol (75 mg).

##### Rutin.

The unhydrolysed aqueous methanolic extract (I) after concentration and standing yielded a yellow precipitate of impure rutin (200 mg) which was crystallized from aqueous ethanol.

The filtrate (I) was extracted immediately with ethyl acetate (VII) and the strong acids removed as outlined in (VIII).

#### 2.1.1.2. E. sieberi.

##### Unknown D.<sup>48</sup>

An off-white precipitate settled in the concentrated ether solution from (II) and was found to be a mixture of flavanones (Hillis' unknown D) together with minor components.

Unknown F.<sup>48</sup>

The ether extract remaining after removal of the above flavanones was extracted repeatedly with 5% aqueous sodium bicarbonate until no appreciable colour showed in the bicarbonate layer. After neutralisation and re-extraction with ether, this extract showed the presence of Hillis' unknown F<sup>48</sup> together with other components.

After several weeks of standing in the cool room a yellow-white precipitate (500 mg) settled in the ether extract of the bicarbonate solubles. A paper chromatogram of this precipitate in two dimensions showed that it contained traces of Hillis' unknown D, later identified as a flavanone mixture, some ellagic acid and Hillis' unknown F.

Trituration of the yellow-white precipitate with ether removed the traces of unknown D. Subsequent repeated trituration with ethyl acetate monitored on two-dimensional chromatograms showed that unknown F was more soluble in ethyl acetate than was ellagic acid so that the triturates gradually concentrated the proportion of unknown F.

However after five triturations and re-crystallizations unknown F could not be obtained pure, ellagic acid persistently re-crystallizing as an impurity.

Unknown A<sup>48</sup>

The aqueous methanolic extract (I) after continuous extraction with ether (II) was then hydrolysed with 2M hydrochloric acid until paper chromatograms showed all glycosides had been ruptured.

This acid hydrolysate was then extracted continuously with ethyl acetate (VII) and n-butanol (XII) as in the General Procedure previously given on pages 21,22.

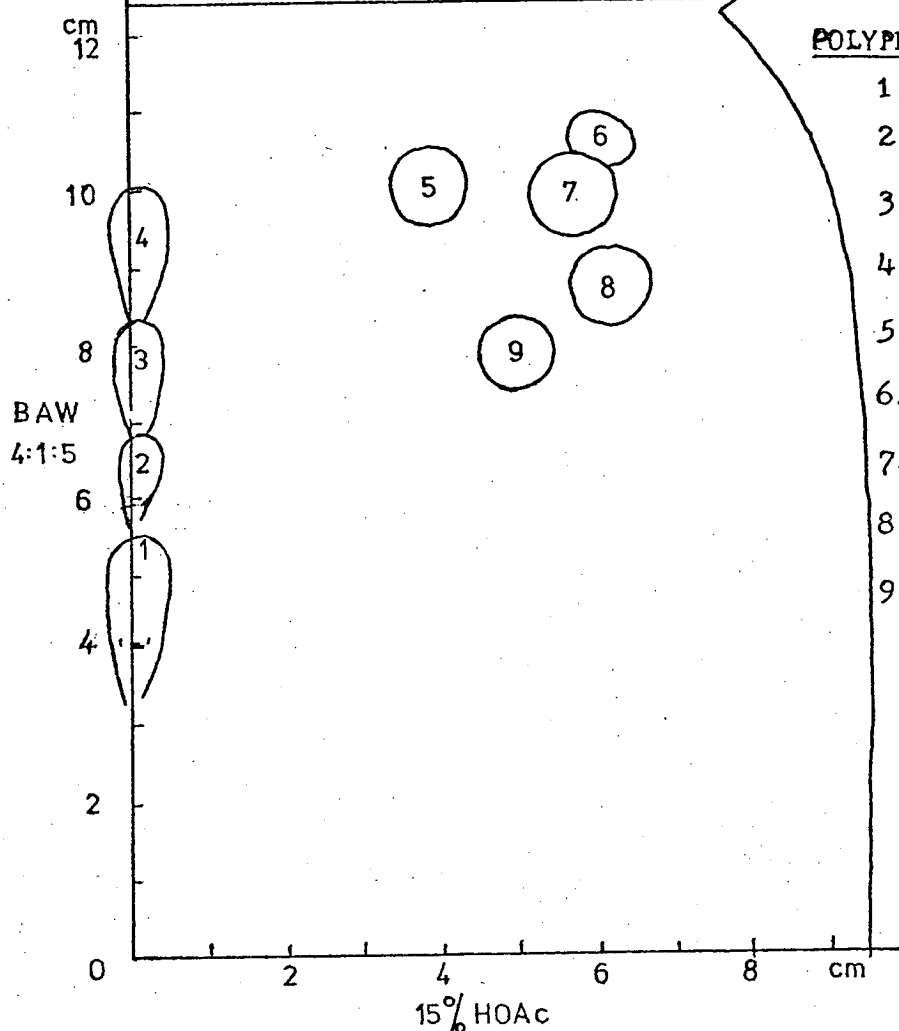
An off-white precipitate in the ethyl acetate extract of the hydrolysed solution after Step (VII) later proved to be a mixture of two components, the minor component being ellagic acid and the major one, Hillis' unknown A.

2.1.1.3. E. coccifera.

Hydroxybenzoic acids, flavonols, ellagic acid,  
Hillis' unknown H.<sup>48</sup>

Portion of the original aqueous methanolic solution was hydrolysed with 2M hydrochloric acid until paper chromatograms showed the hydrolysis of glycosides was complete, the solution becoming (IH) for the liquid-liquid extractions in Charts 5,6, pages 21,22.

Figure 1 shows that the ether extract of the neutralised bicarbonate solution contained caffeic (Spot 5), gallic (9), gentisic (6), chlorogenic (8) and ellagic acids (1) together with a mixture of kaempferol (4), quercetin (3) and myricetin (2) and finally a spot which would correspond to Hillis' unknown H (7).

Fig.1. POLYPHENOLS of *E.coccifera* extracted by 5%  $\text{NaHCO}_3$ 

POLYPHENOL	365nm	$\text{NH}_3$ 365nm
1.	mauve	dull yellow
2.	orange	intense or.
3.	yellow	intense y.
4.	y-gr.	intense y-gr.
5.	blue-wh.	blue-white
6.	blue-wh.	bl-gr-white
7.	pink	orange
8.	blue	intense green
9.	mauve (254nm)	dark mauve (254nm)

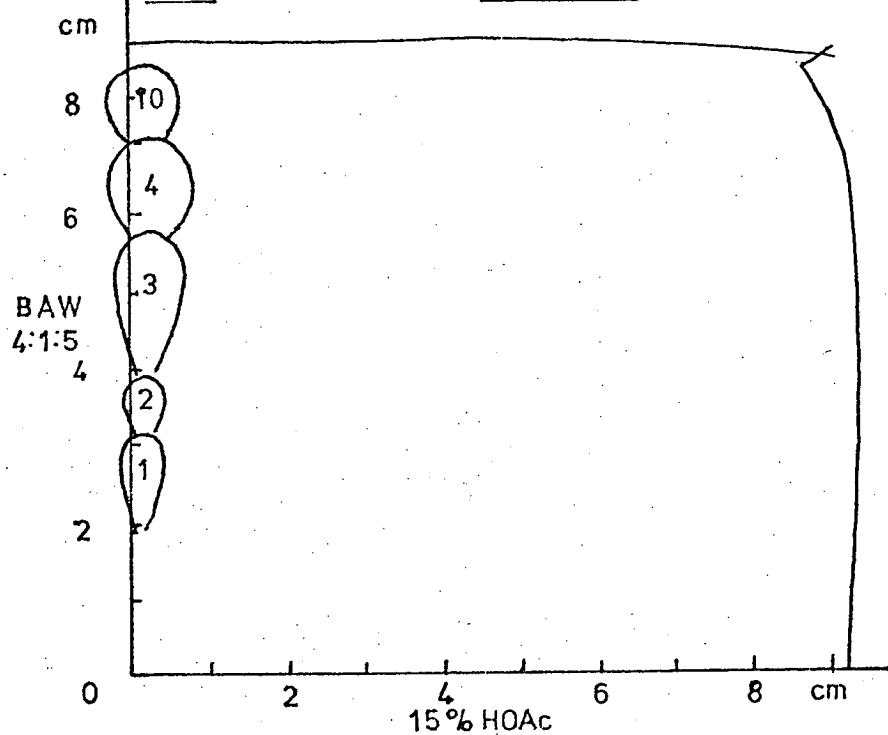
Abbreviations:

or. = orange

y. = yellow

y-gr. = yellow-green

blue-wh. = blue-white

bl-gr-white = blue-  
green-white.Fig.2. POLYPHENOLS in *E.coccifera* ETHER EXTRACT after  $\text{NaHCO}_3$  extraction.

POLYPHENOL	365nm	$\text{NH}_3$ 365nm
1.	mauve	dull yellow
2.	orange	intense or.
3.	yellow	intense y.
4.	y-gr.	intense y-gr.
10.	purple	purple-blue

Abbreviations:

or. = orange

y. = yellow

y-gr. = yellow-green

48  
Unknown D.

The ether solution from (III), i.e. after bicarbonate extraction of it, was shown on paper chromatograms (Figure 2) to contain a mixture of compounds, mainly unknown D (Spot 10), a flavonol mixture (4,3,2) and ellagic acid (1).

Glycosides.

A second portion of the original aqueous methanolic solution (I) was carried through the liquid-liquid extractions in Charts 5,6, pages 21,22, without hydrolysis.

Figure 3 shows a paper chromatogram of the ether extract from Step (II) in Chart 5.

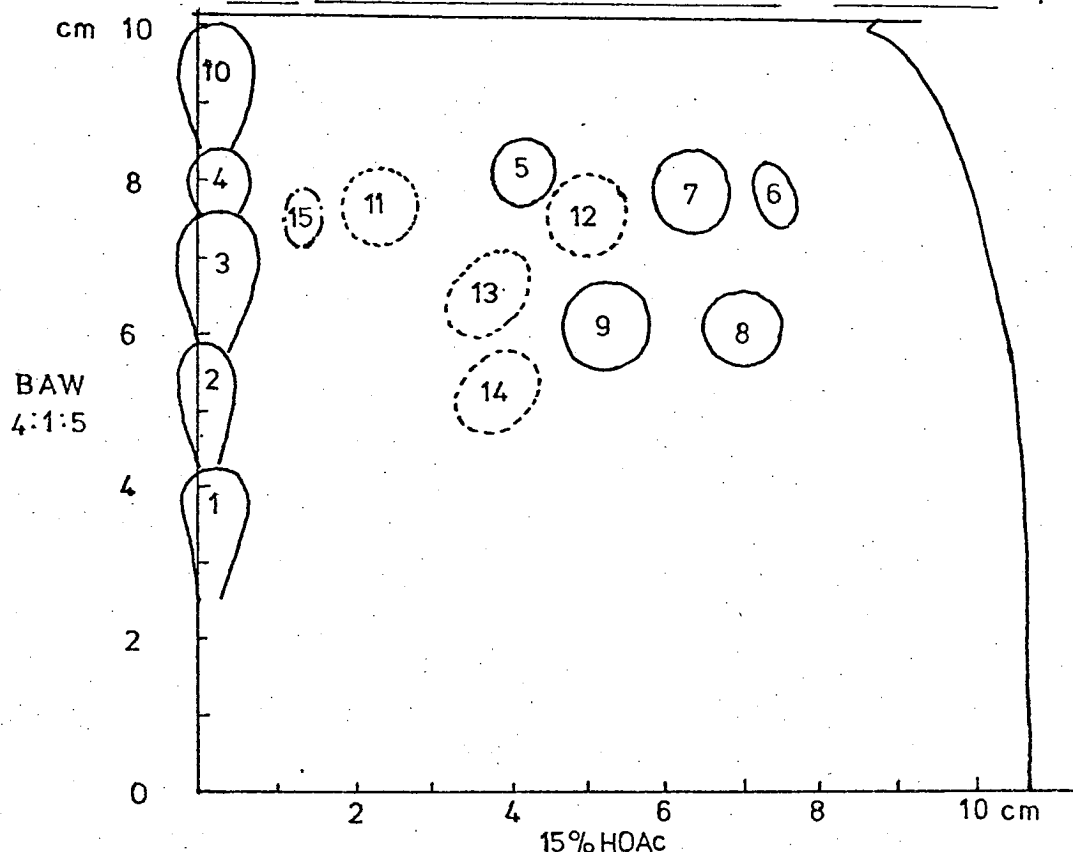
2.1.2. CHROMATOGRAPHY.

Applications of the chromatographic materials listed on pages 23-26 are now given in more detail for each species examined.

2.1.2.1. E. delegatensis.

Extracts chromatographed are referred to by numerals corresponding to Charts 5,6 on pages 21,22.

The ether extract of neutralised bicarbonate solution (V) was reduced to dryness and dissolved in 3% aqueous acetic acid. This mixture was applied to a cellulose column and eluted first with 3% then 6% aqueous acetic acid. Gentisic and protocatechuic acids were eluted as a mixture (50 mg) followed by impure gallic acid (50 mg). Quercetin, unknown F and ellagic acid were removed only when eluted with 50% aqueous ethanol.

Fig.3. POLYPHENOLS in ETHER EXTRACT of *E.coccifera* aq:MeOH solution.

<u>POLYPHENOL</u>	<u>15% HOAc</u> <u>colour (365nm)</u>	<u>colour/NH<sub>3</sub> (365nm)</u>
1. ellagic acid	mauve	dull yellow
2. myricetin	orange	intense orange
3. quercetin	yellow	intense yellow
4. kaempferol	yellow-green	intense yellow-green
5. caffeic acid	blue-white	white-blue
6. gentisic acid	blue-white	blue-green-white
7. Hillis' Unknown H	pink	orange
8. chlorogenic acid	blue	intense green
9. gallic acid	dark mauve (254nm)	intense dark mauve (254nm)
10. Hillis' Unknown D	purple-blue	purple-blue
11. afzelin	purple	yellow-green
12. quercitrin	purple	yellow
13. isoquercitrin	purple	yellow
14. hyperin	purple	yellow
15. sinapic acid (trace)	blue-green	light-green

Column eluates were monitored on paper chromatograms which showed that while gentisic and protocatechuic acids had approximately identical  $R_f$  values when developed with 6% acetic acid, they had different  $R_f$  values in BAW (415).

Repeated preparative paper chromatography on Whatman No 3 paper using BAW (415) as developing solvent, with drying between developments, isolated sufficient gentisic (35 mg) and protocatechuic acids (20 mg) for their identification.

The ether extract remaining after extraction with bicarbonate (VI) was concentrated and a portion (2 ml) applied to a preparative thickness cellulose thin layer plate and the plate developed with CEF solvent yielding impure quercetin (100 mg), which crystallized as yellow needles from dilute alcohol (85 mg), and a compound (7 mg) corresponding in chromatographic properties to Hillis' unknown D<sup>48</sup> crystallized as white needles from methanol (3 mg).

The unhydrolysed methanolic aqueous extract (I) after concentration and standing yielded a yellow precipitate of impure rutin (200 mg) which crystallized from dilute alcohol.

The ethyl acetate solution after bicarbonate extraction (XI) was taken to dryness onto polyamide powder, which was applied to a polyamide column eluting first with water and then with ethanol-water mixtures



of increasing ethanol concentration. Bands were observed in u.v. light and the ethanol concentration increased until a satisfactory movement of bands was achieved.

The 30-70% ethanol eluates were combined, evaporated to dryness and portion of the residue dissolved in aqueous acetic acid after which it was streaked onto six sheets of Whatman No 3 paper and developed three times with 6% aqueous acetic acid drying the papers after each development. The papers yielded impure quercitrin (30 mg) which crystallized as yellow needles from water (25 mg).

A small quantity of a second quercetin glycoside (5 mg) was also eluted from these papers from a band having a smaller  $R_f$  than quercitrin and later identified as isoquercitrin, after crystallization from water.

The 80-100% ethanol eluates were evaporated to dryness and the residue chromatographed two-dimensionally showing the presence of rutin and a spot initially interpreted as gallic acid but soon detected as a catechin isomer by its colours when sprayed with vanillin in hydrochloric<sup>13</sup> and ethanolic p-toluene sulphonic acids.<sup>96</sup>

The 80-100% ethanol eluates residue was dissolved in n-butanol and extracted with borate buffer (pH 8.2) until no colour appeared in the buffer. On acidification,

concentration and standing the buffer extract yielded a precipitate of impure catechin isomer (90 mg) which crystallized from dilute acetic acid (70 mg).

2.1.2.2. E. sieberi.

Preparative paper chromatography of the bicarbonate extractives (III) using the descending method in 6% aqueous acetic acid separated the glycosides (20 mg) from other polyphenols with a minimum of hydrolysis.

Dissolved in methanol the mixture of glycosides was spotted onto sheets of Whatman No 1 paper and the sheets developed in BAW (415) in the ascending direction with the chromatograms being dried and re-developed in the same solvent a second time. The same papers were then developed in the second dimension using 15% aqueous acetic acid as developing solvent and again the development was repeated.

In this way the separation of the glycosides shown in Chart 7 was achieved.

Spots with corresponding  $R_f$  values were eluted with ethanol containing 30% water and the eluate evaporated and spotted on Whatman No 1 paper for final purification by two-dimensional development in the same solvents as above.

Final elution and evaporation yielded glycosides which after crystallization were identified as quercitrin (4 mg), isoquercitrin (4 mg), hyperin (3 mg) and cannabiscitrin (3 mg).

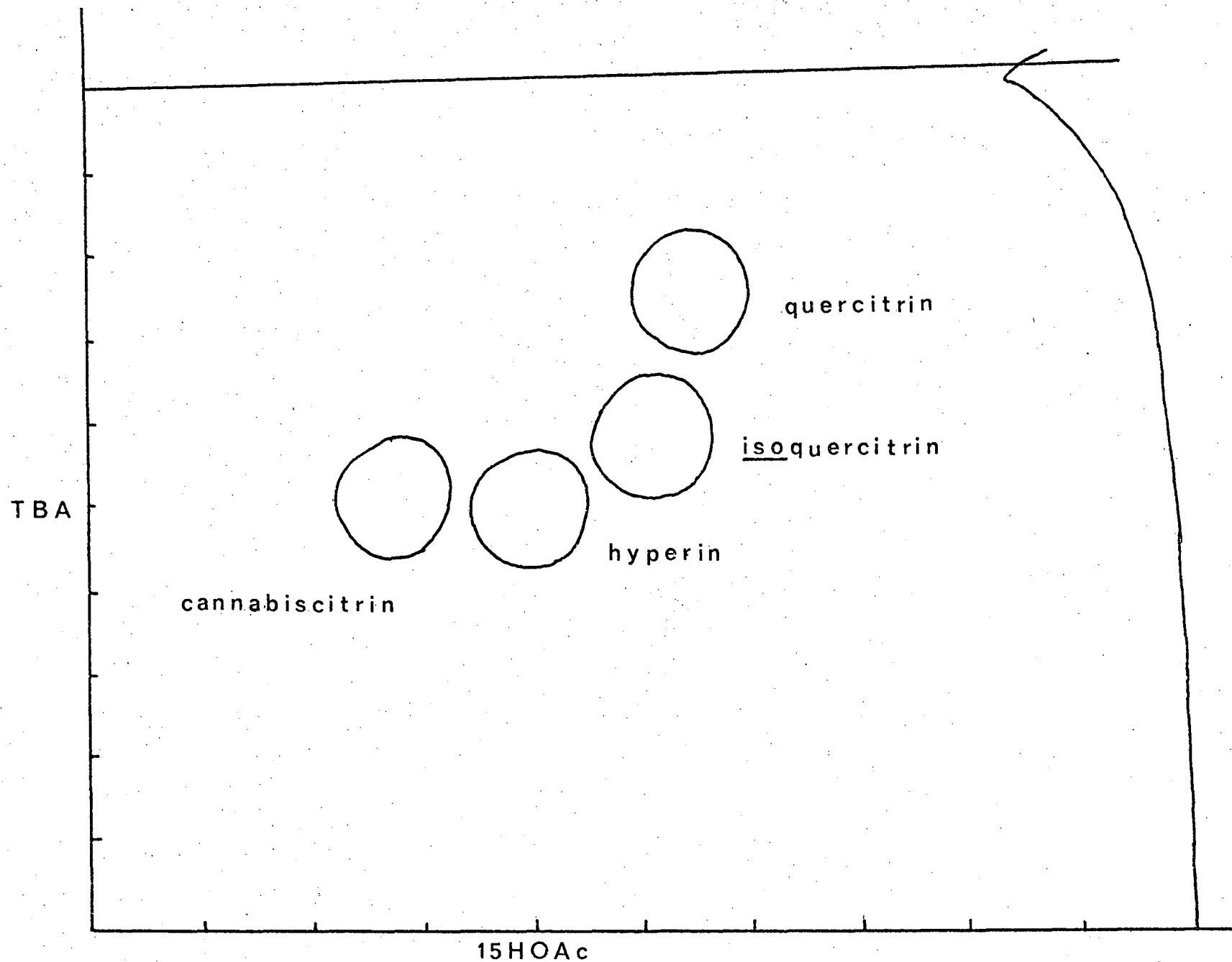


CHART 7. Chromatogram of *E. sieberi* glycosides.

The butanol extract obtained from Step (XII) was extracted five times with borate buffer of pH 8 and then by 5% aqueous sodium bicarbonate solution. Chromatographic survey of the neutralised bicarbonate solution which had been re-extracted into butanol showed the probable presence of quercetin, myricetin as well as gallic, gentisic and ellagic acids (Chart 8, overleaf).

After evaporation under vacuum the crude mixture shown in Chart 8 (350 mg) was dissolved in methanol, applied to a Sephadex LH 20 column and was allowed to be adsorbed onto the gel before elution began using ethanol as eluent. Fractions were monitored on thin layer chromatoplates using TEF (541) as solvent.

After bulking of like fractions the separation of compounds obtained is shown in Chart 9, page 39.

The compounds isolated from the Sephadex column followed by preparative t.l.c. and crystallization are listed in Table 3.

TABLE 3.

<u>Fractions.</u>	<u>Compound isolated.</u>	<u>Crystallizing solvent.</u>
1-14	gallic acid (50 mg)	methanol.
15-30	quercetin (60 mg)	aqueous ethanol.
31-50	gentisic acid (30 mg)	methanol.
51-200	quercetin (23 mg)	aqueous ethanol.
201-240	myricetin (18 mg)	aqueous ethanol.
241-300	ellagic acid (95 mg)	aqueous ethanol.

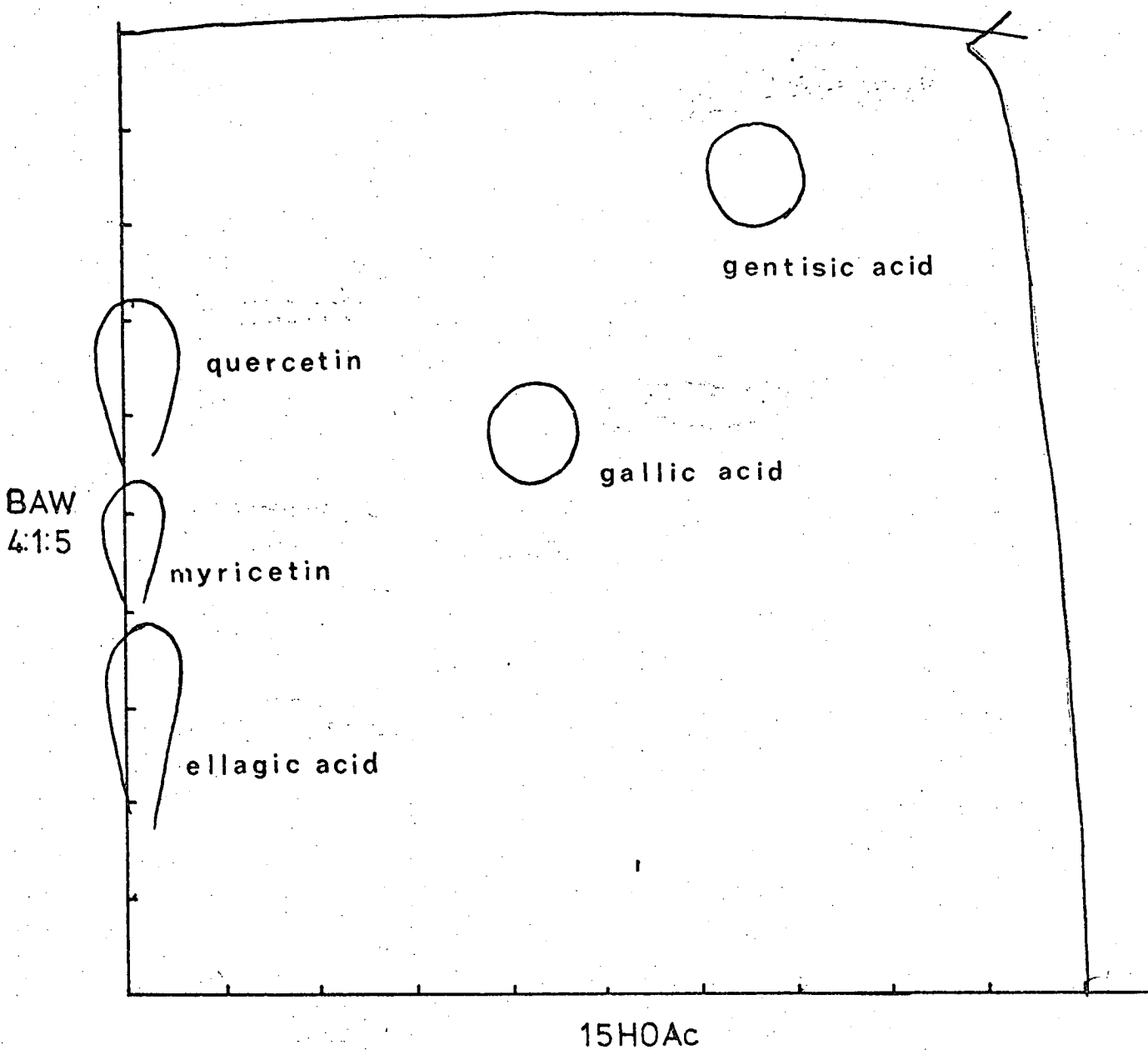


CHART 8. Chromatogram of n-butanol extract of neut.  $\text{NaHCO}_3$  solution.

SEPHADEX LH 20 COLUMN

TLC  
SILICA

toluene : ethyl acetate : formic acid  
5 : 4 : 1

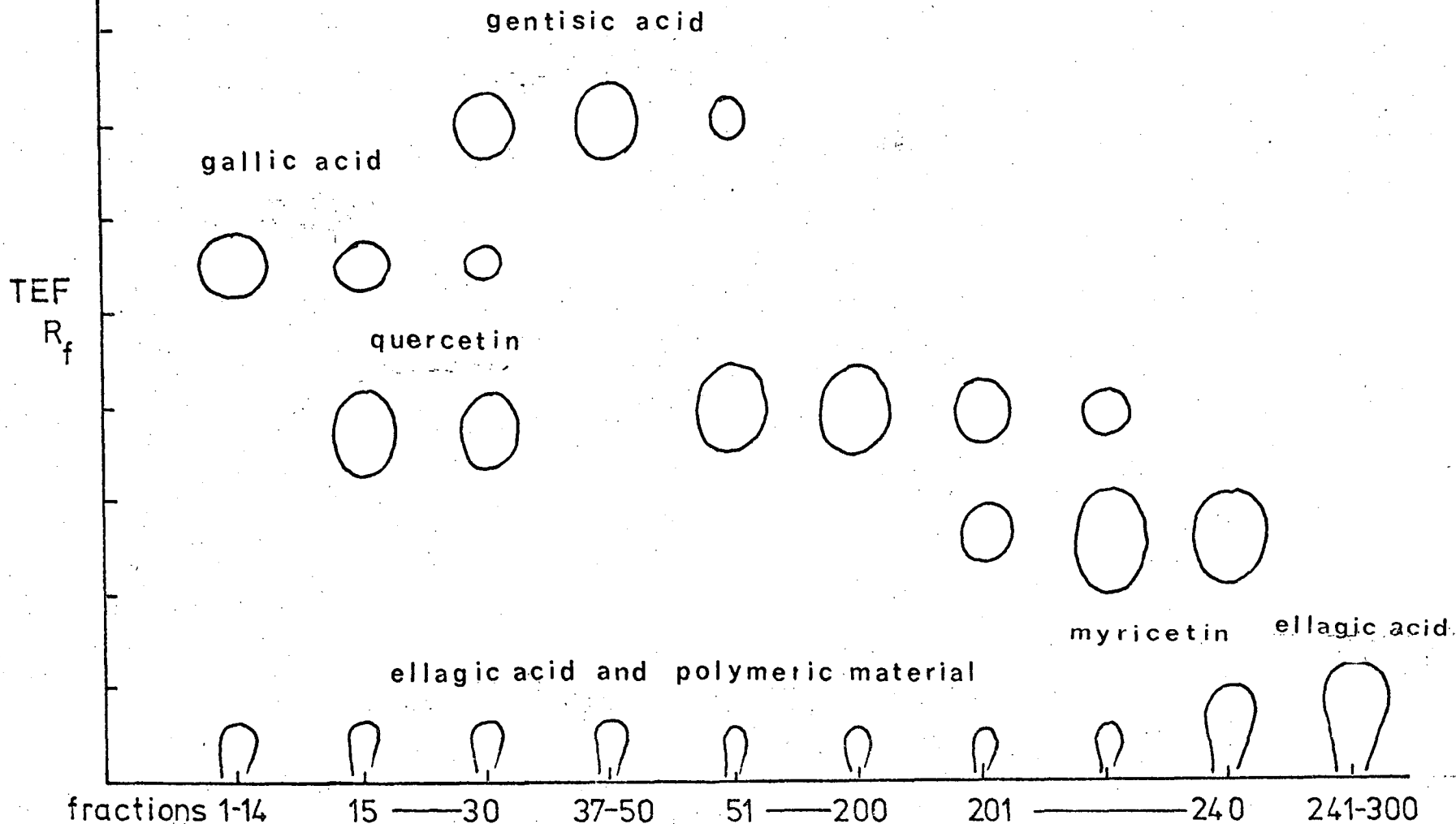


CHART 9. SEPHADEX COLUMN ELUATES CHROMATOPLATE.

An off-white precipitate (350 mg) settled in the ethyl acetate extract (VII) of the hydrolysed solution which had been left to stand in the refrigerator.

Paper chromatography showed this precipitate to be in the main a mixture of two components, the minor component being ellagic acid and the major component having colours in u.v. light similar to those of Hillis' unknown A<sup>48</sup> although not having identical  $R_f$  values, possibly because of slightly different solvent systems.

A chromatoplate of silica gel to which the mixture (125 mg) had been applied was developed with the 15HOAC solvent system. In this way unknown A was separated from ellagic acid and crystallized from aqueous ethanol to give a pure compound (60 mg) which has been identified as 4-O-monomethyl ellagic acid.

The ethyl acetate extract remaining after removal of the above precipitate was paper chromatographed and showed in addition to the above compounds the presence of hydroxybenzoic acids and a spot with properties similar to that of Hillis' unknown B.<sup>48</sup>

Repeated extraction of the ethyl acetate solution with 5% aqueous sodium bicarbonate with subsequent neutralisation of the bicarbonate solution showed by two-dimensional chromatograms that the unknown B compound had been extracted by the bicarbonate extraction.

Re-extraction of the neutralised bicarbonate

solution with ethyl acetate and evaporation was followed by application of the mixture (150 mg) to a silica plate developed with BPF (36:9:5). The top band of the plate contained impure unknown B.

After elution and taking to dryness, the component in the top band (45 mg) was applied to sheets of Whatman No 3 MM paper which were developed with 15% aqueous acetic acid, dried and re-developed with the same solvent until the characteristic diffuse mauve bands of unknown B were resolved.

Elution of these bands with aqueous ethanol and evaporation gave a residue which crystallized from aqueous ethanol to give a compound (28 mg) with chromatographic properties similar to those of unknown B.

Whatman cellulose powder was formed into a small column, the unknown F mixture obtained by trituration of an ether precipitate was applied in 15% aqueous acetic acid and this solvent used in column development. Fractions collected showed on paper chromatograms that separation had still not been achieved.

Finally a portion (150 mg) of the unknown F mixture was applied to a silica chromatoplate using CEF as developing solvent. Paper chromatogram monitoring showed there had not been a clean separation but there seemed a greater concentration of unknown F in two bands which were eluted, evaporated and re-run several times.



In this way sufficient unknown F (16 mg) was obtained after three re-crystallizations to obtain spectral analyses.

2.1.2.3. E. coccifera.

Roman numerals used in the following paragraphs refer to Charts 5,6 on pages 21,22.

The ether solution remaining after extraction with bicarbonate (VI) was shown by paper chromatography to contain a mixture of unknown D, flavonols and ellagic acid.

The unknown D mixture spots on paper did not give red colours when sprayed with sodium borohydride and subsequently exposed to acid vapour<sup>33</sup> as the E. sieberi flavanones had done. This prompted the isolation of the E. coccifera unknown D mixture.

Analytical thin layer chromatography using Camag silica gel as adsorbent, and chloroform; ethyl acetate; formic acid (7:4:1, CEF) as solvent suggested that column chromatography using the same adsorbent and developing solvent might separate the unknown D mixture.

The ether after bicarbonate extraction was taken to dryness (3 g) and this was dissolved in CEF (7:4:0.5), placed on a silica column and developed and eluted with the same solvent system. Fractions collected were monitored on analytical t.l.c. plates with CEF (7:4:1) as developing solvent system.

Figure 4 summarises the separation after fractions of similar composition had been joined.

Fraction A contained a mixture of apparently two compounds fluorescing purple under u.v. light (365 nm) and a minor component of higher  $R_f$  value fluorescing blue under u.v. light. Preparative t.l.c. (silica, CEF) then separated the blue component from the purple components mentioned above.

The compounds in Fraction A which fluoresced purple were separated into two spots on analytical silica plates using CEF as solvent.

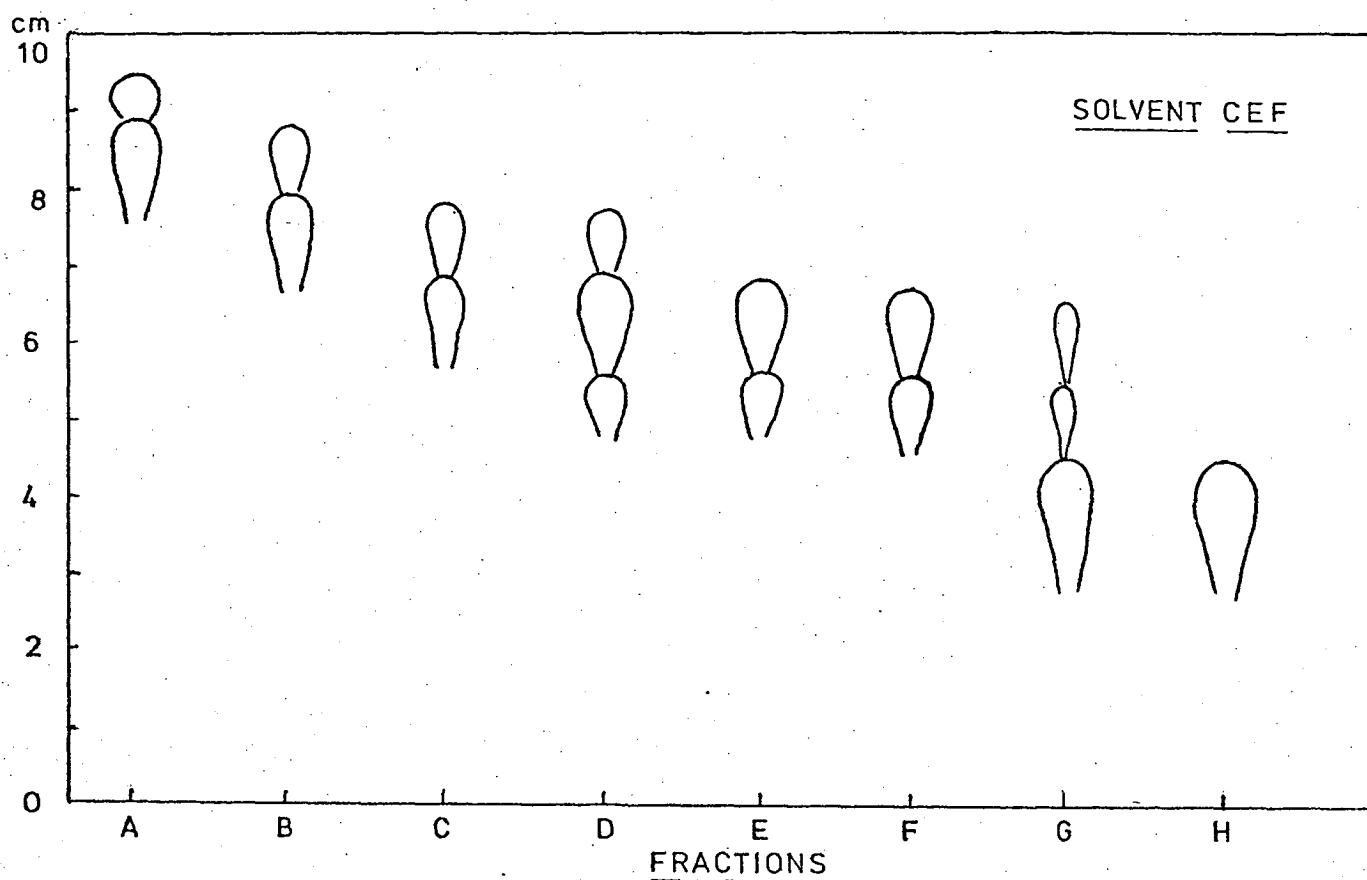
Preparative t.l.c. using the same adsorbent and solvent separated the purple-fluorescent materials into two bands which when eluted gave apigenin as the band of lower  $R_f$  and what appeared to be another single compound of higher  $R_f$ .

In all, six solvent systems were used before one of them, BPF, showed that in fact the apparently single compound was a mixture of a major and minor component.

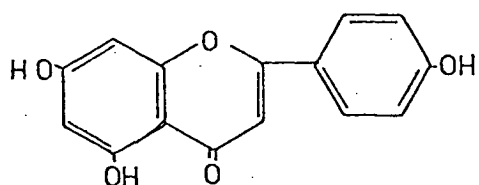
Figure 5 shows the analytical t.l.c. separation of this mixture using BPF, into components later identified as (4), (5) and (6).

It now appeared that the purple fluorescing spot in Fraction A was a mixture of three compounds, one of lower  $R_f$  whose isolation has already been mentioned (apigenin (4)) and a mixture of two presumably

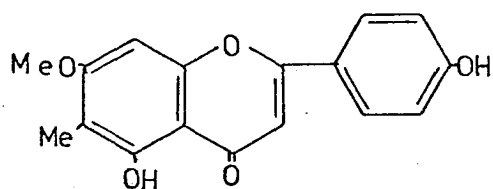
Fig.4. SILICA COLUMN SEPARATION of E.coccifera POLYPHENOLS.



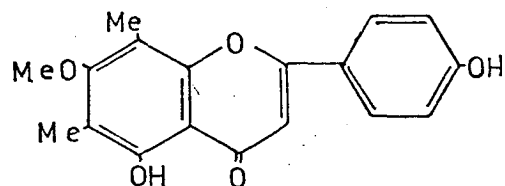
- A. Unidentified compound and Unknown D mixture.
- B. Unknown D mixture and kaempferol
- C. Kaempferol and quercetin
- D. Kaempferol, quercetin and myricetin
- E. Quercetin, myricetin
- F. Quercetin, myricetin
- G. Traces of quercetin, myricetin and ellagic acid
- H. ellagic acid



(4)



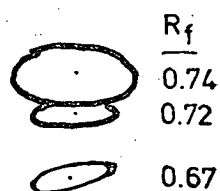
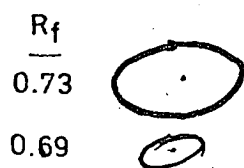
(5)



(6)

E.coccifera FLAVONES.

Fig.5. TLC SEPARATION of FLAVONE MIXTURE  
USING CEF and BPF SOLVENTS.



CEF  
CHCl<sub>3</sub>:EtOAc:HCOOH  
3 : 4 : 1  
7 : 4 : 1

BPF  
C<sub>6</sub>H<sub>6</sub>:C<sub>6</sub>H<sub>5</sub>N:HCOOH  
6 : 6 : 5 : 5  
36 : 9 : 5

closely related compounds of higher  $R_f$ .

Repeated preparative t.l.c. on silica using BPF and involving the procedure of developing, drying and re-developing, separated the major component of the mixture. Several crystallizations from ethanol gave a chromatographically pure compound, identified on evidence recorded in the discussion section as a rare C-methylflavone, found only once before in nature, sideroxylin or 4',5-dihydroxy-7-methoxy-6,8-dimethyl flavone (25 mg) (6).

The minor component (5) in this mixture was present in too small an amount to enable isolation in sufficient quantity for complete spectral analysis. However enough material (0.1 mg) was obtained by eluting spots from six analytical silica chromatoplates developed three times with BPF for u.v. spectral analysis, discussed in Chapter 4, page 150.

Fraction C from the column was shown by two-dimensional paper chromatography to be a mixture mainly of kaempferol and quercetin. Kaempferol was isolated using preparative silica t.l.c. plates with MCP as developing solvent; kaempferol (20 mg) crystallized from aqueous ethanol.

An ether extract of the original unhydrolysed aqueous methanolic solution (I) contained four glycosides.

Quercitrin, isoquercitrin and hyperin were identified using known markers on paper chromatograms.

The remaining glycoside, different from others previously found in this work, was isolated by preparative paper chromatography by repeated two-dimensional development using first 6HOAc and then BAW (612) as developing solvents.

In this way sufficient of this glycoside was obtained (2 mg) to identify it as afzelin.

---

### CHAPTER 3.

#### IDENTIFICATION - EXPERIMENTAL.

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### CHAPTER 3.

#### 3.0. SPECTRAL DETERMINATIONS.

##### 3.0.1. U.v. SPECTRA.

3.0.1.1. Stock diagnostic reagents used throughout this work and prepared according to the methods of Mabry et al.<sup>75b</sup> were sodium methoxide (NaOMe), aluminium (III) chloride ( $\text{AlCl}_3$ ), hydrochloric acid (HCl), sodium acetate (NaOAc) and boric acid ( $\text{H}_3\text{BO}_3$ ). The reagents above were applied and spectra run according to the procedures given in detail by the same authors.

3.0.1.2. U.v. spectra were run on a Hitachi Perkin-Elmer 124 double beam spectrophotometer or on a Perkin-Elmer 400 spectracord in methanol alone or in methanol with the reagents given above; stock solutions of the polyphenols being prepared in either of the following ways:<sup>75b</sup>

3.0.1.3. The solutions used were made from re-crystallized compound (0.1 mg) in Analar methanol (10 ml) and the concentration adjusted so that maximum absorbance between 240-420 nm occurred between 0.6 - 0.8. When log  $\epsilon$  values were obtained the concentration was accurately determined.

3.0.1.4. Alternatively, the polyphenols were purified by one or two-dimensional paper chromatography. Spots, when sufficiently discrete, were viewed under u.v. light, their zones cut out of the chromatogram and eluted with Analar methanol. The compound was dried by evaporation,

re-dissolved in a minimum of methanol and its spectrum was recorded as above.

Since methanol may elute u.v.-absorbing compounds from the paper itself, when spectra were run in this way a reference solution was made by extracting a piece of blank chromatogram which had been developed in the same way as the chromatogram containing the polyphenol(s).

### 3.0.2. N.m.r. SPECTRA.

N.m.r. spectra were recorded on a Jeolco JNM4H-100 spectrometer at 100 MHz or on a Varian A60 spectrometer at 60 MHz for 5% solutions in DMSO-d<sub>6</sub> with HMDS or TMS as external standard or in the case of the 60 MHz spectra, as internal standard, or using C<sub>5</sub>D<sub>5</sub>N or CDCl<sub>3</sub> or mixtures of the first two and the last, with TMS as internal standard. In the case of spectra run to analyse trimethylsilyl ethers of polyphenols a drop each of hexamethyldisilazane and trimethylchlorosilane was added to ensure anhydrous conditions.<sup>75c</sup> It was standard procedure to run additional spectra after addition of deuterium oxide for detection of hydroxyl groups.

### 3.0.3. I.r. SPECTRA.

These were determined in Nujol and hexachlorobutadiene mulls or using KBr discs, on a Perkin-Elmer 221 spectrophotometer.

### 3.0.4. MASS SPECTRA.

Mass spectra were measured on an Ae.I.MS9 instrument at 70 eV. or on an EA1 QUAD 300 quadrupole mass spectrometer.

### 3.1. SPECTRA.

Spectra are presented in two sections, first (3.2.1.), those referring to polyphenols of known structure and the second (3.2.2.), those of polyphenols of unknown structure (Hillis' unknowns).<sup>48</sup>

The spectra given are referred to in discussion in Chapter 4.

#### 3.1.1. SPECTRA OF POLYPHENOLS OF KNOWN STRUCTURE.

##### 3.1.1.1. Hydroxybenzoic acids.

Chart 10. N.m.r. spectra of gallic, gentisic and protocatechuic acids.

11. Mass spectra.

##### 3.1.1.2. Flavonols.

Chart 12. N.m.r. spectrum of quercetin.

13. N.m.r. spectrum of quercetin compared with that of myricetin.

14. Mass spectra.

15. Mass fragmentation.

3.1.1.3. Ellagic acid.

- Chart 16. Mass spectrum and fragmentation.
17. N.m.r. spectrum ( $C_5D_5N/EtOH$ ).
18. N.m.r. spectrum of TMS ether  
( $CDCl_3$ ).

3.1.1.4. (+)-Catechin.

- Chart 19. N.m.r. spectrum ( $DMSO-d_6$ ).
20. N.m.r. spectrum ( $C_5D_5N$ ).
21. Mass fragmentation.
-

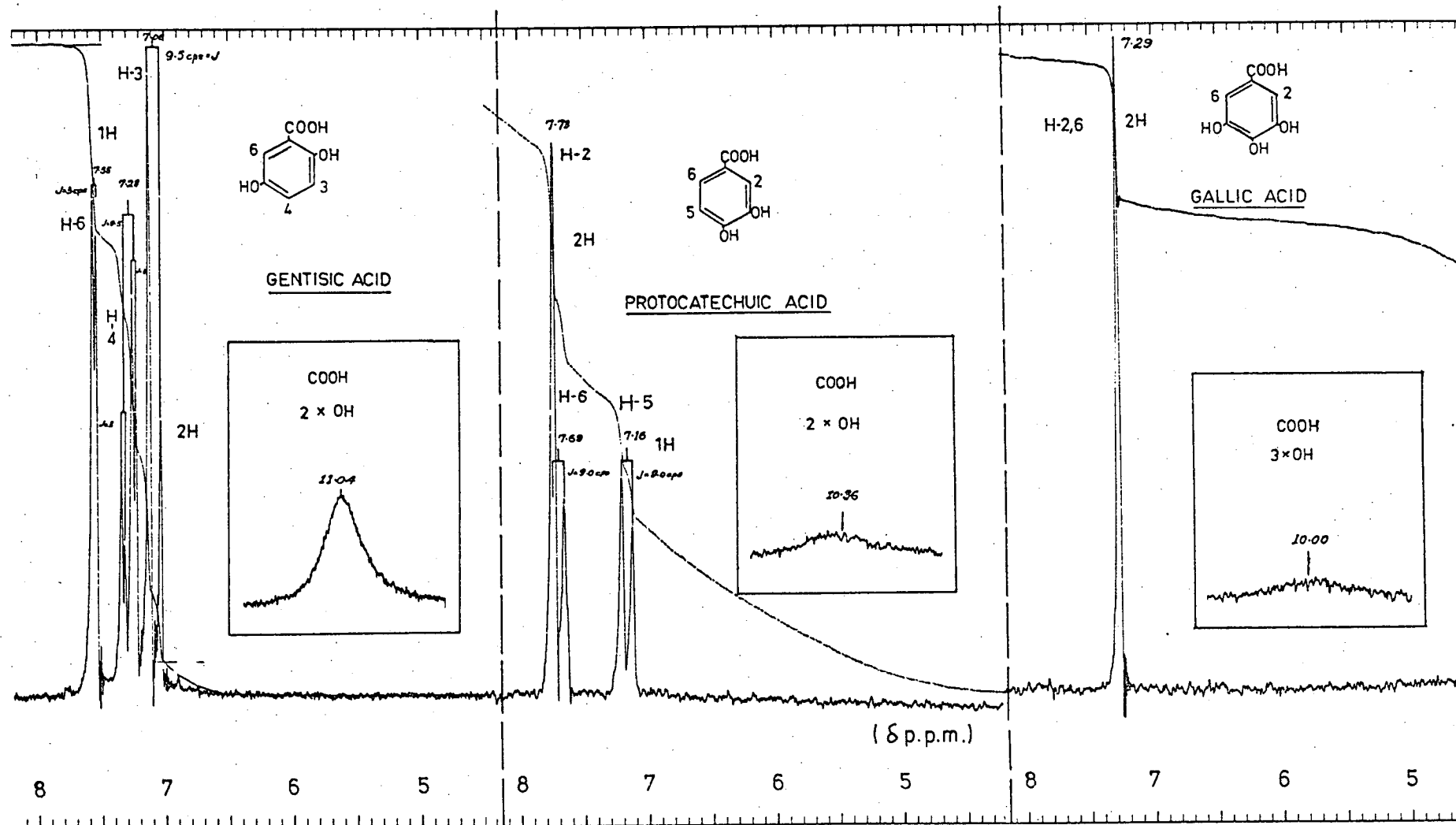


CHART 10. N.M.R. SPECTRA of HYDROXYBENZOIC ACIDS.

GENTISIC ACID

PROTOCATECHUIC ACID

GALLIC ACID

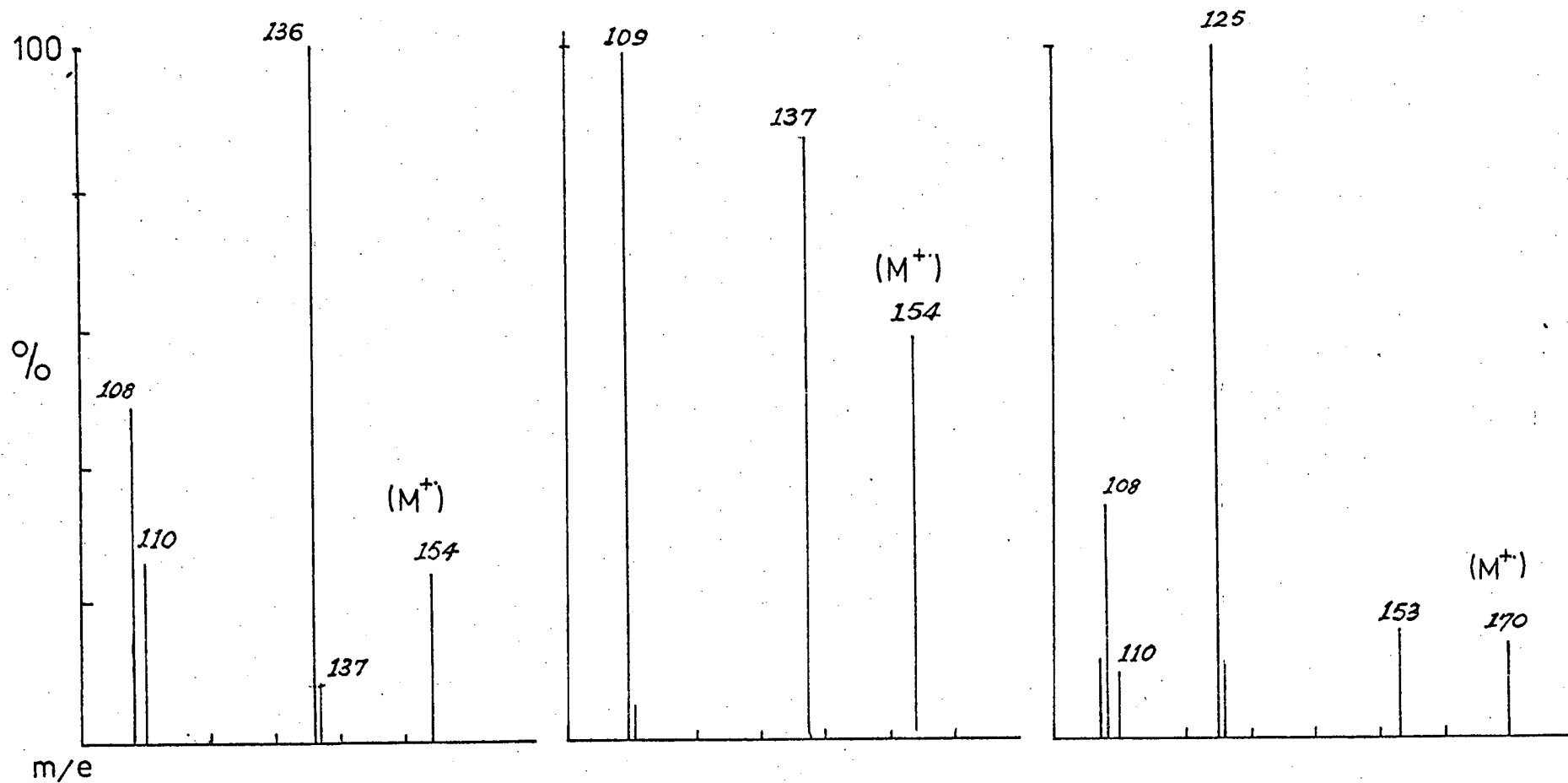
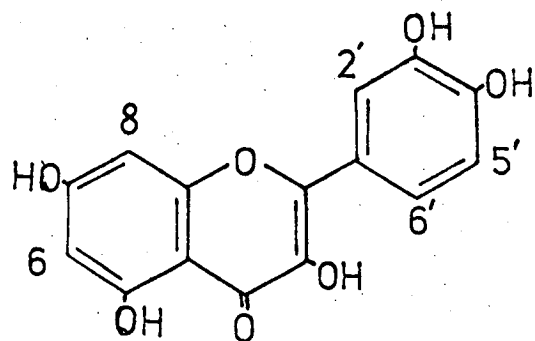


CHART 11. MASS SPECTRA of HYDROXYBENZOIC ACIDS.



QUERCETIN

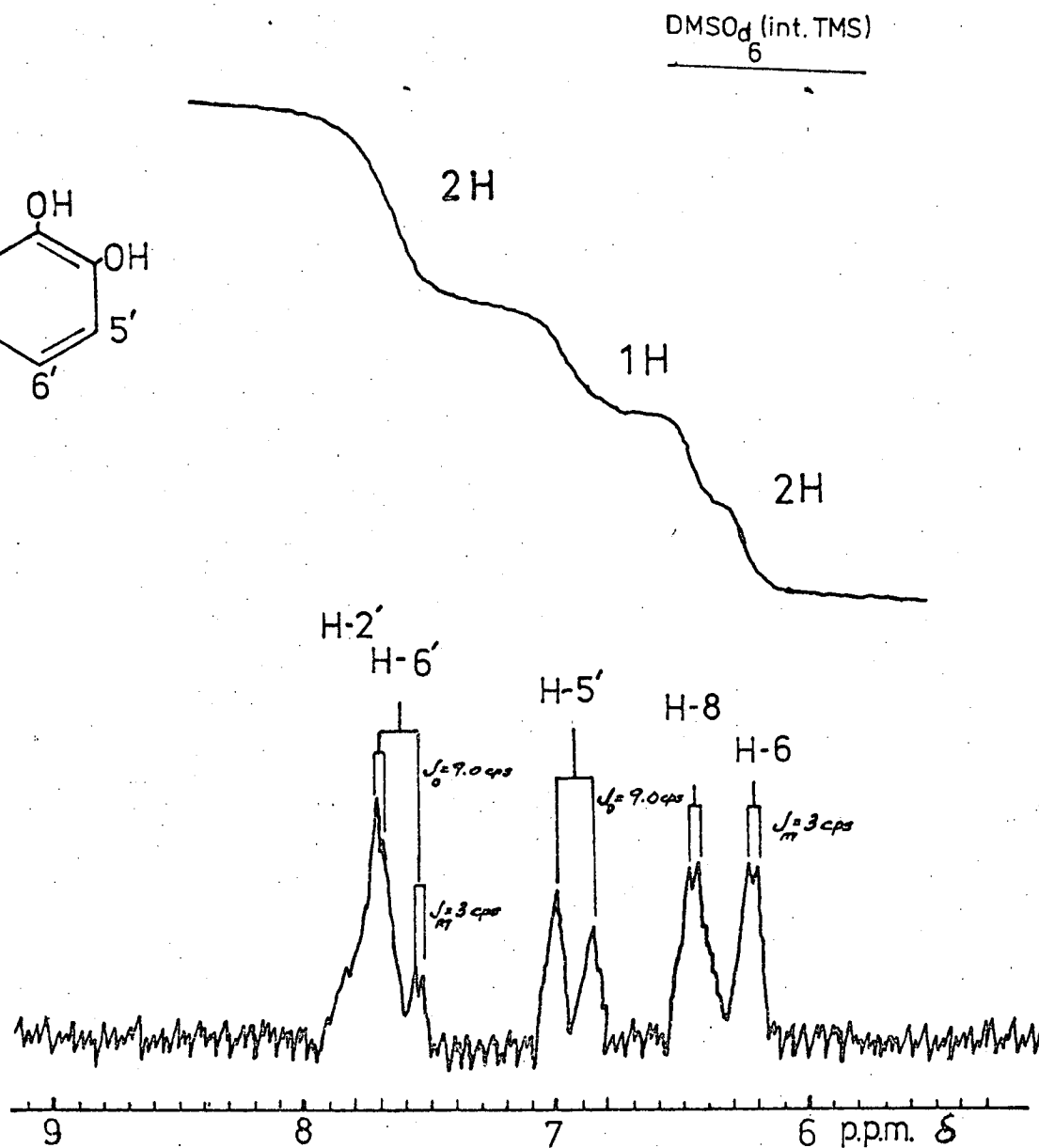
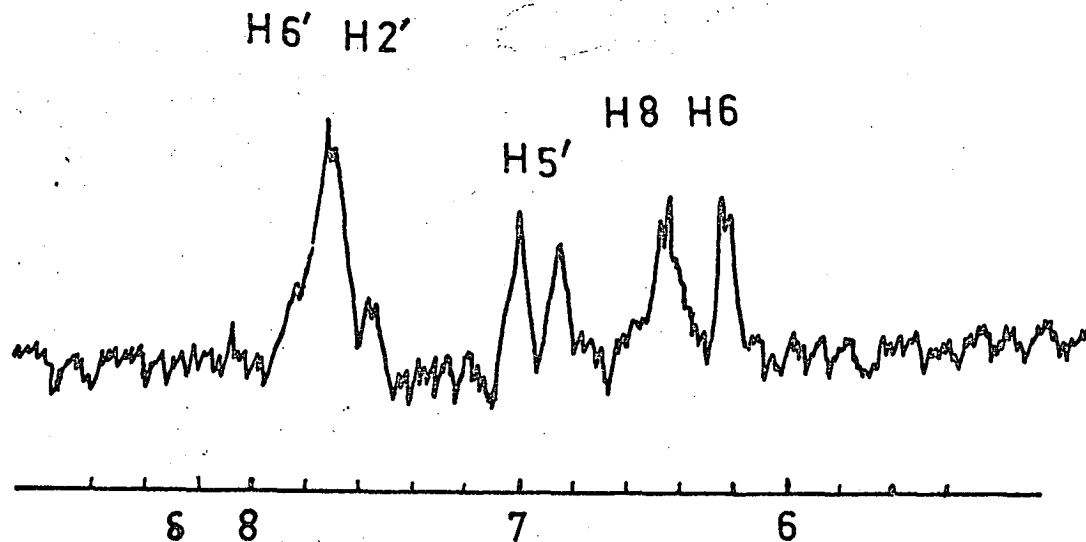
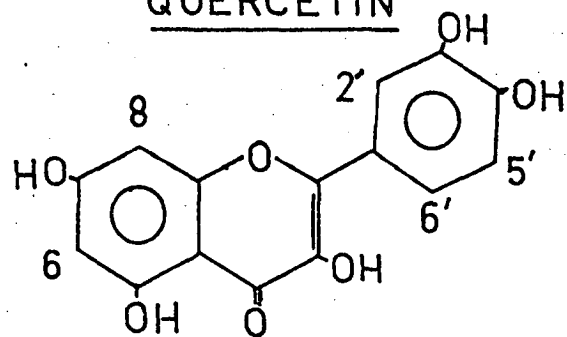


CHART 12. N.M.R. SPECTRUM of QUERCETIN.

QUERCETIN



MYRICETIN

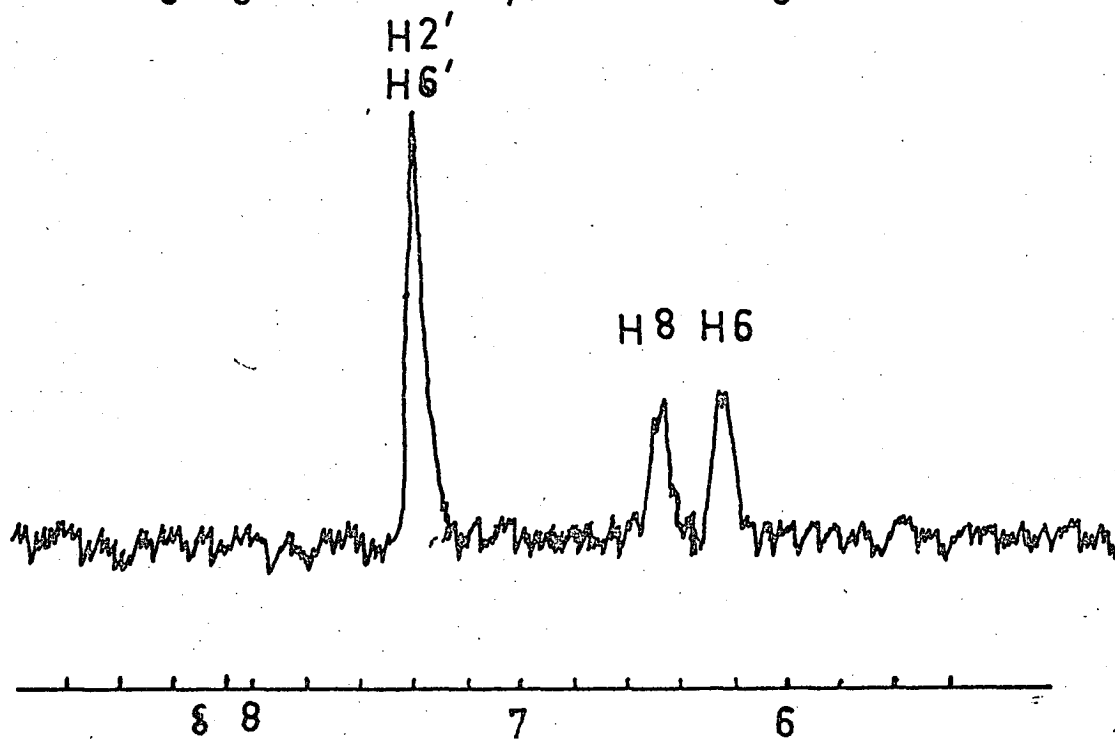
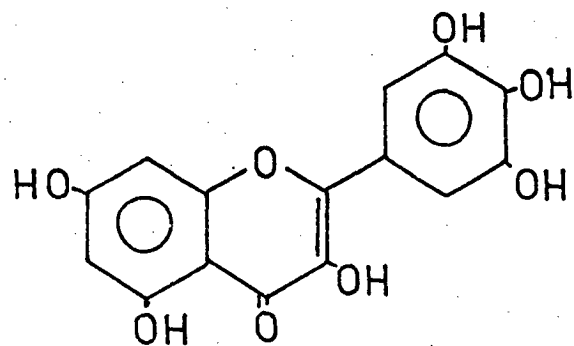


CHART 13.

N.M.R. SPECTRA of FLAVONOLS.



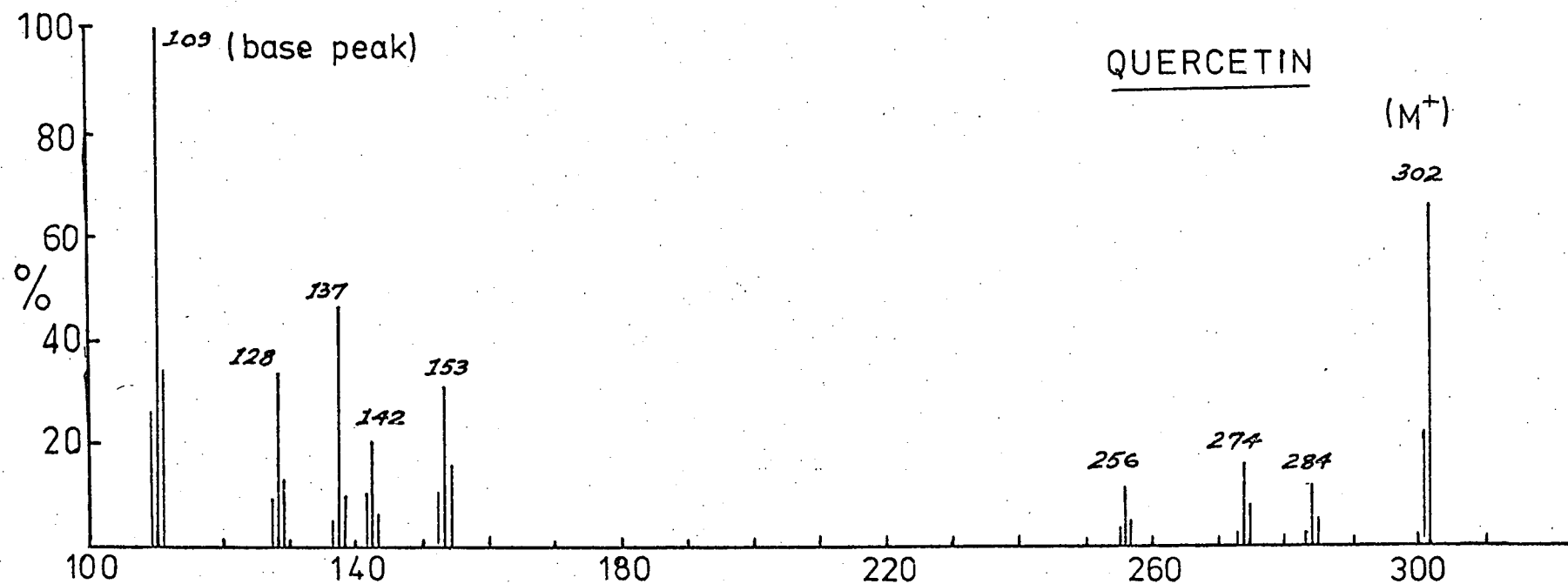
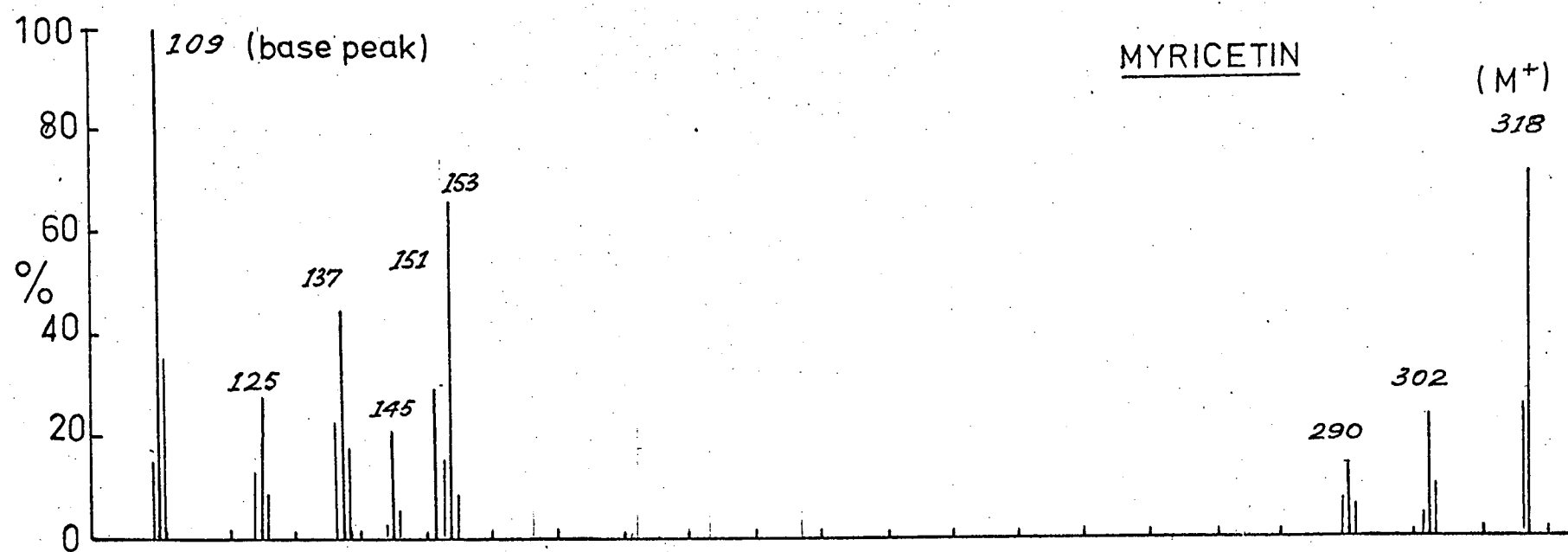


CHART 14. MASS SPECTRA of E.sieberi FLAVONOLS.

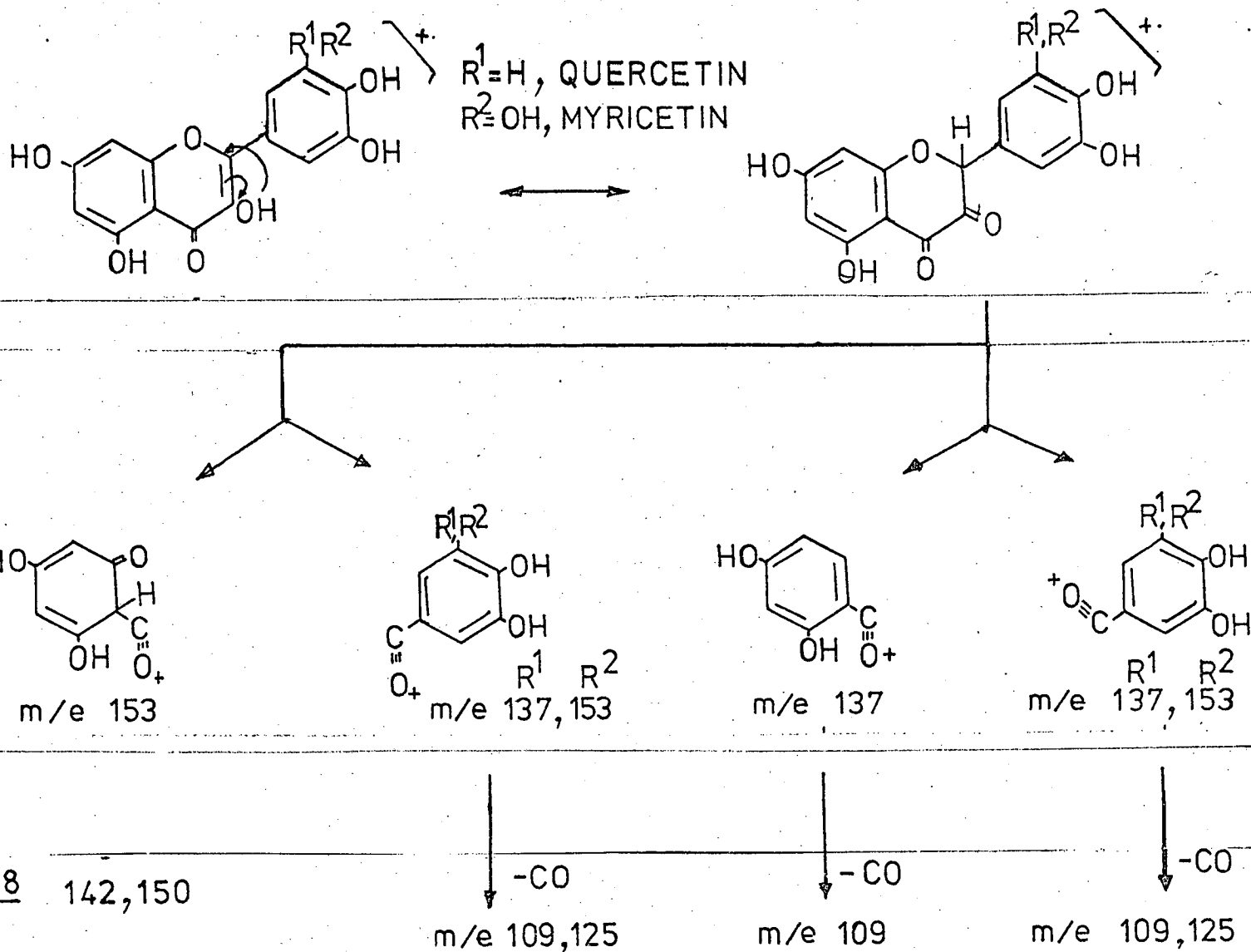


CHART 15. MASS FRAGMENTATION of *E.sieberi* FLAVONOLS.

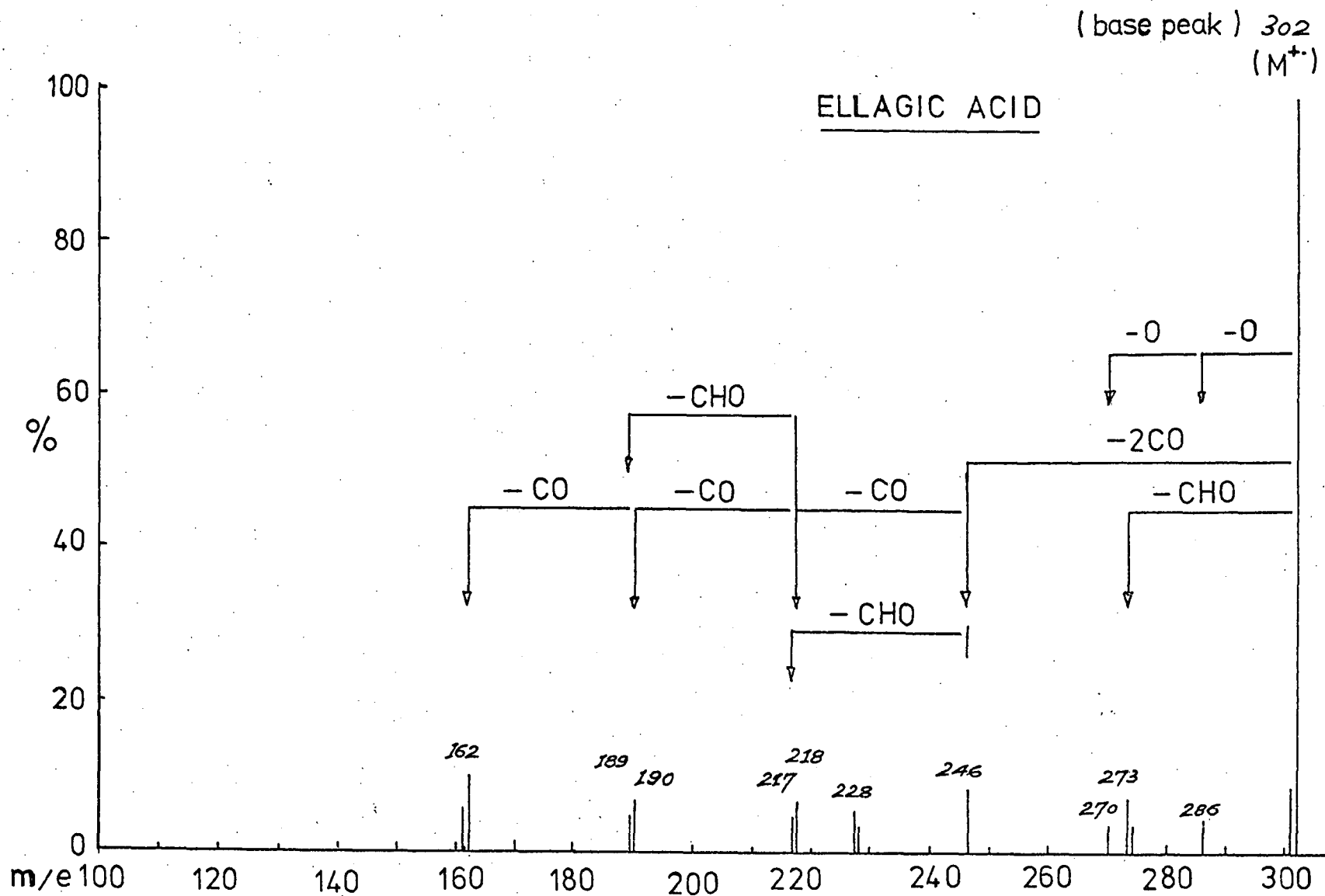


CHART 16. MASS SPECTRUM and FRAGMENTATION of ELLAGIC ACID

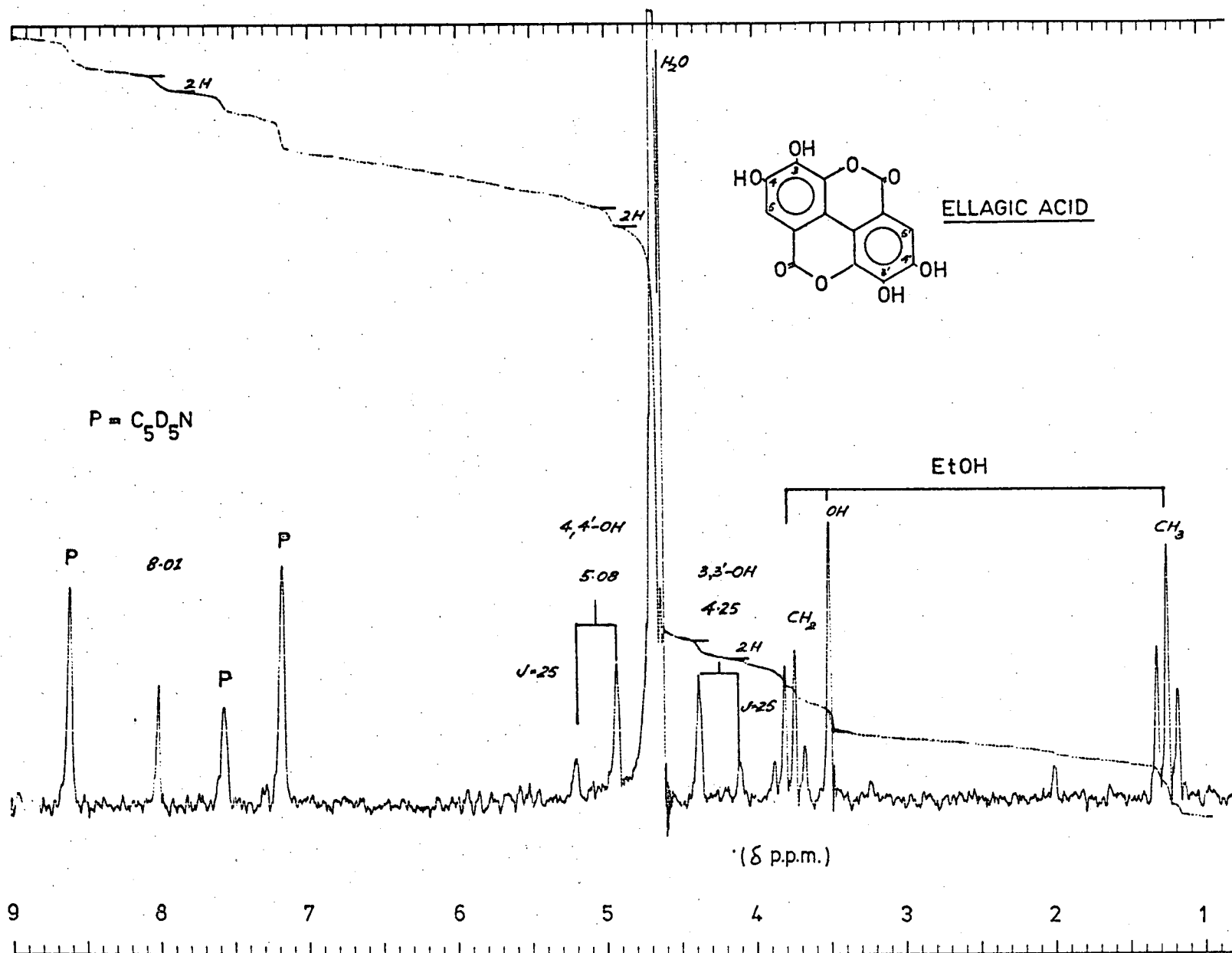


CHART 17. N.M.R. SPECTRUM of ELLAGIC ACID.

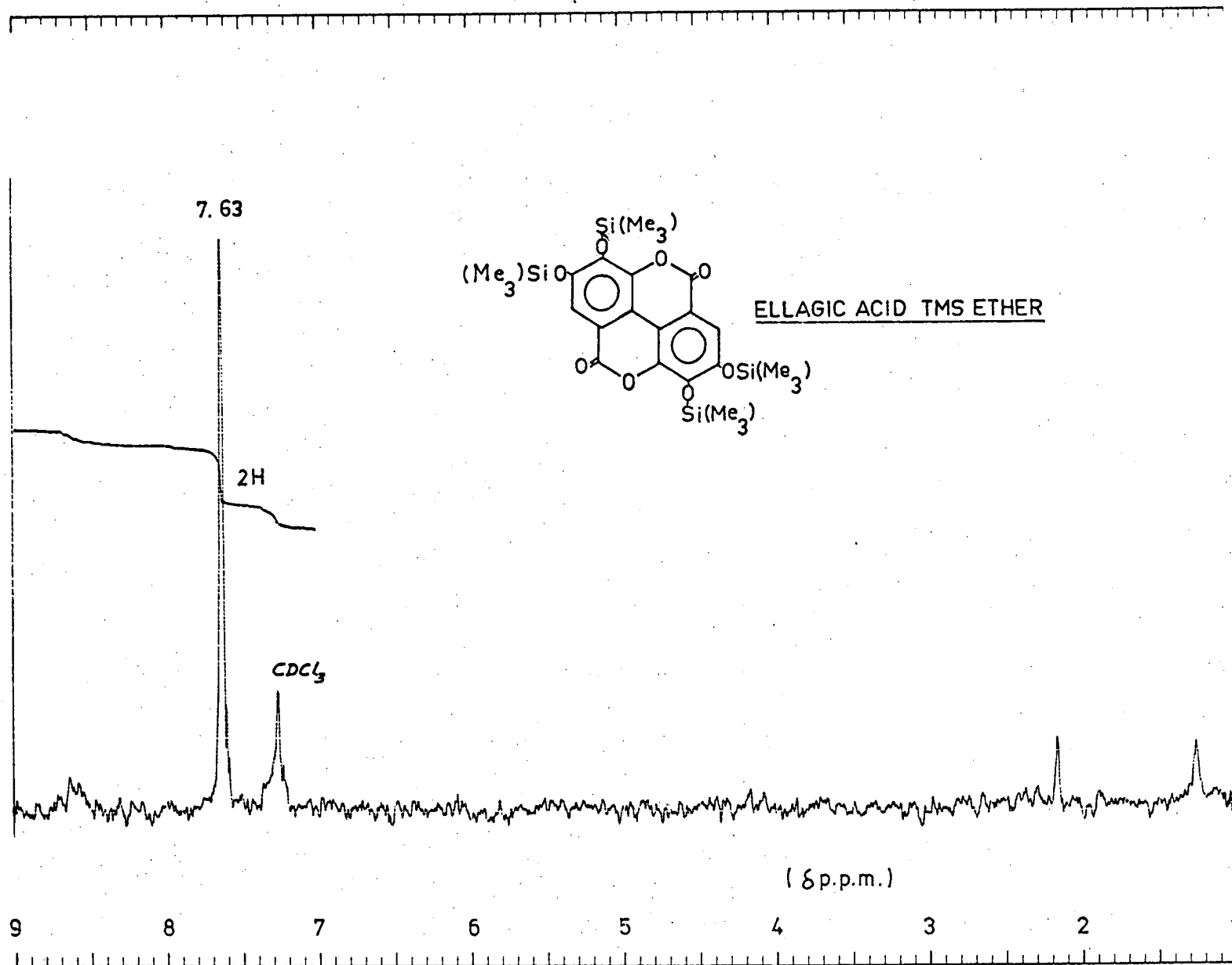
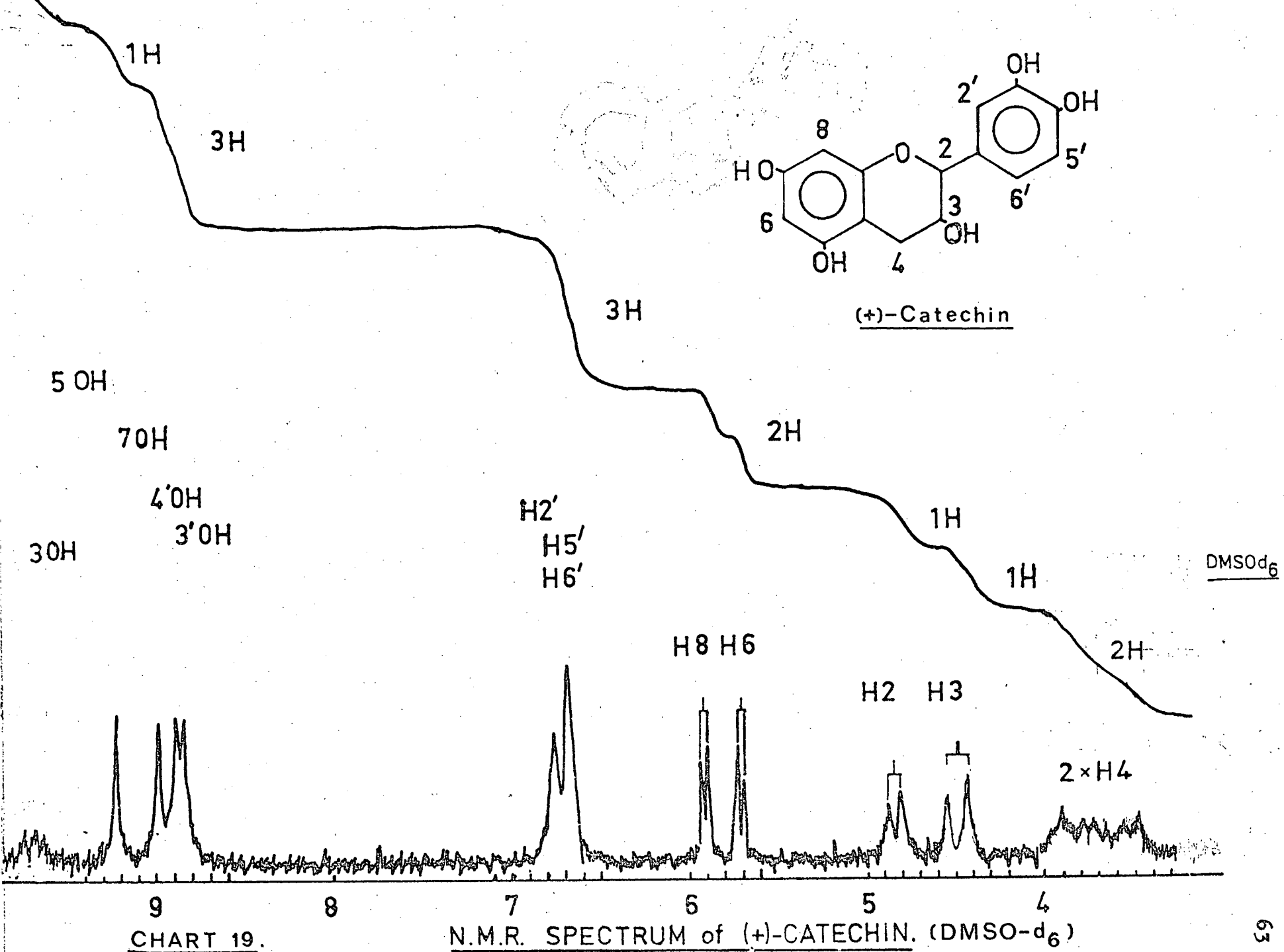


CHART 18. N.M.R. SPECTRUM of ELLAGIC ACID TMS ETHER.





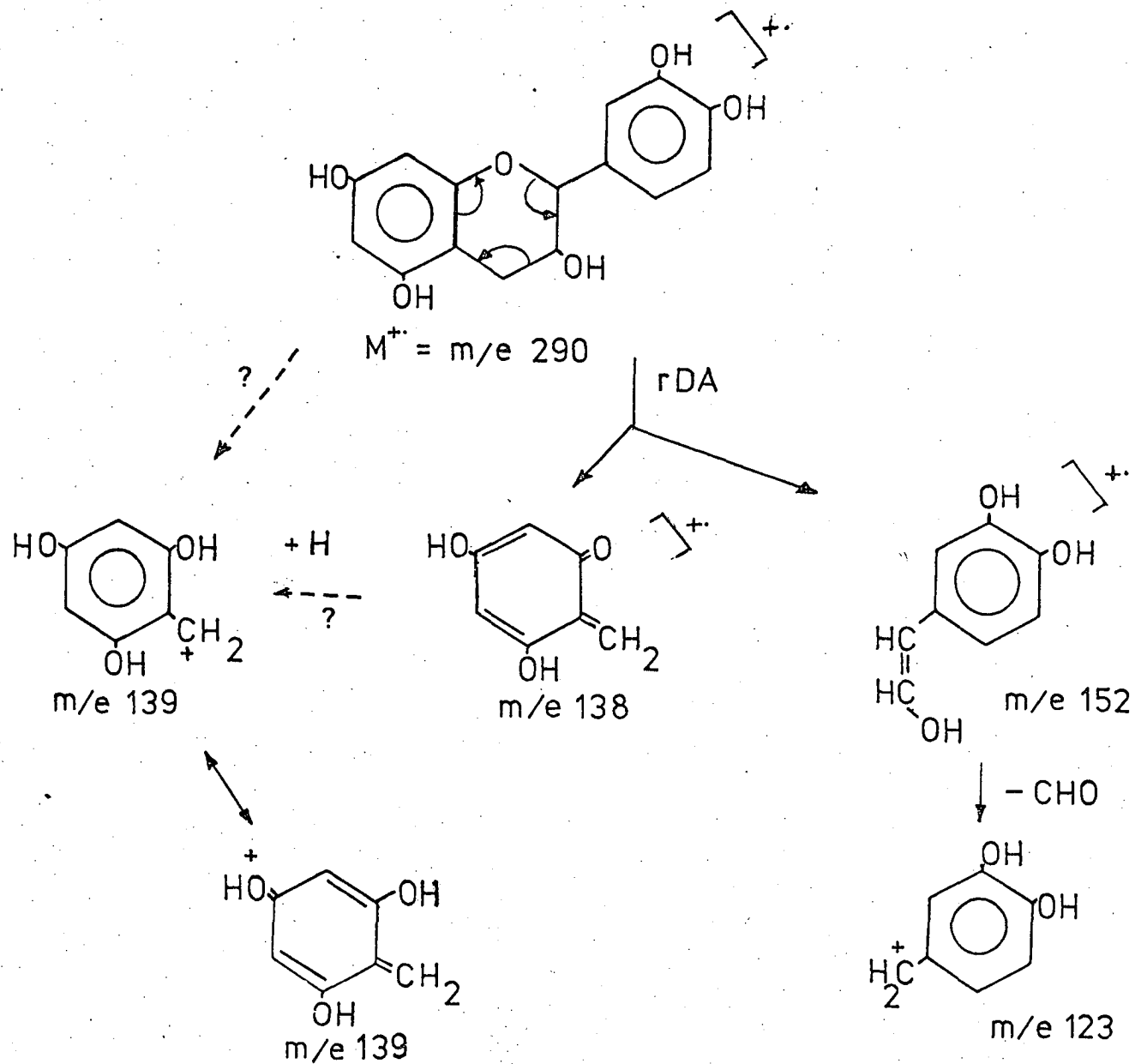


CHART 21. MASS FRAGMENTATION of (+)-CATECHIN.



### 3.1.2. SPECTRA OF POLYPHENOLS OF UNKNOWN STRUCTURE.

#### 3.1.2.1. Unknown D<sup>48</sup> - Flavanones.

- Chart 22. N.m.r. spectra and configuration.
23. I.r. spectral data.
24. N.m.r. spectral data.
25. N.m.r. spectrum of pinocembrin.
26. N.m.r. spectrum of alpinetin.
27. N.m.r. spectrum of O,O-dimethyl-pinocembrin.
28. Mass spectra.
29. Mass fragmentation.

#### 3.1.2.2. Unknown D<sup>48</sup> - Flavones.

- Chart 30. N.m.r. spectrum of apigenin.
31. Mass spectrum of apigenin.
32. Mass fragmentation of apigenin.
33. Mass spectrum of sideroxylin.
34. Mass fragmentation of sideroxylin.
35. N.m.r. spectrum of sideroxylin  
(DMSO-d<sub>6</sub>/CDCl<sub>3</sub>).
36. N.m.r. spectrum of sideroxylin  
(C<sub>5</sub>D<sub>5</sub>N/CDCl<sub>3</sub>).
37. N.m.r. spectrum of sideroxylin mixture.
38. N.m.r. spectrum of TMS ether of  
sideroxylin mixture.
39. Mass fragmentation of second  
C-methyl flavone.
40. Mass spectrum of sideroxylin mixture.

3.1.2.3. Unknown A<sup>48</sup> - 4-O-methyl ellagic acid.

- Chart 41. N.m.r. spectrum.
42. N.m.r. spectrum of methylated unknown A ( $\text{CDCl}_3$ ).
43. N.m.r. spectrum of methylated unknown A ( $\text{C}_6\text{D}_6$ ).
44. U.v. spectra of unknown A and ellagic acid in methanol.
45. U.v. spectra of the same in methanol plus solid sodium acetate.
46. U.v. spectra of the same in methanol plus sodium methoxide solution.
47. Mass spectrum and fragmentation of unknown A.

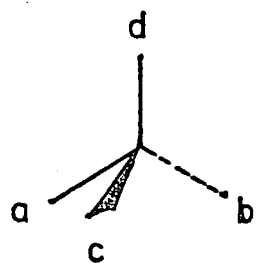
3.1.2.4. Unknown B<sup>48</sup> - (1,5-dimethyl-2,6-bis(3,4,5-trihydroxyphenyl) furo [1,5-c] furan.).

- Chart 48. U.v. spectrum of unknown B in methanol.
- U.v. spectrum in methanol plus sodium methoxide solution.
49. Mass spectrum.
50. N.m.r. spectrum.

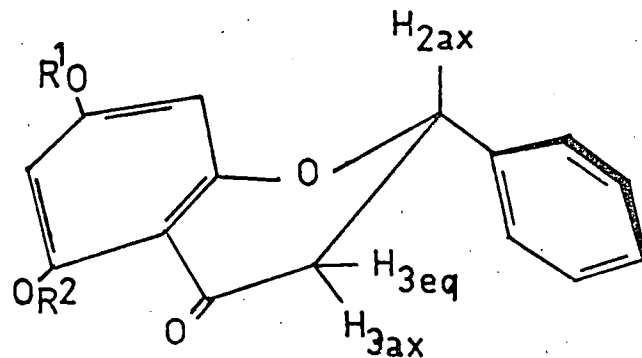
3.1.2.5. Unknown F<sup>48</sup> - (a tetrahydroxydibenzofuran-dicarboxylic acid).

- Chart 51. N.m.r. spectrum.
52. I.r. spectra of ellagic acid and unknown F.
53. Mass spectrum.

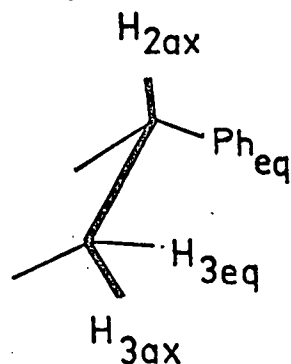
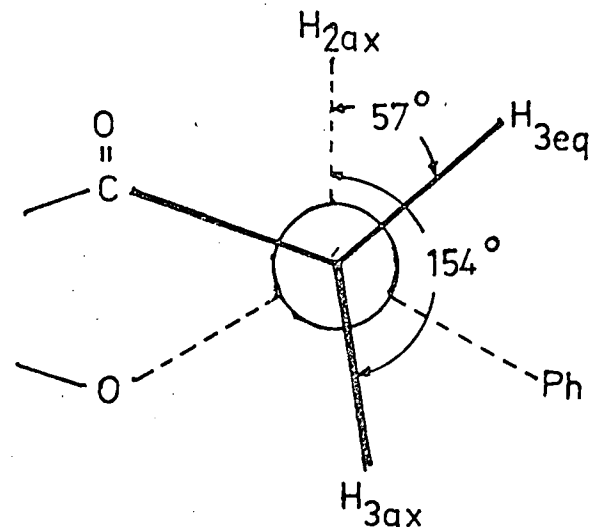
# NMR SPECTRA and CONFIGURATION of *E.sieberi* FLAVANONES.



2S

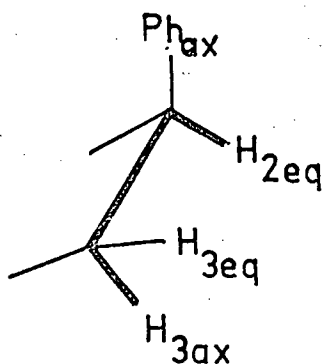


## DIHEDRAL ANGLES



$H_{ax} - H_{ax}$

$J \doteq 12 \text{ Hz}$



$H_{eq} - H_{ax}$

$J \doteq 2.5 \text{ Hz}$

OBSERVED  $J \doteq 13 \text{ Hz}$

$$J_{H2-3ax} \doteq 16 \cos^2 154^\circ \doteq 13.0 \text{ Hz}$$

$$J_{H2-3eq} \doteq 10 \cos^2 57^\circ \doteq 3.0 \text{ Hz}$$

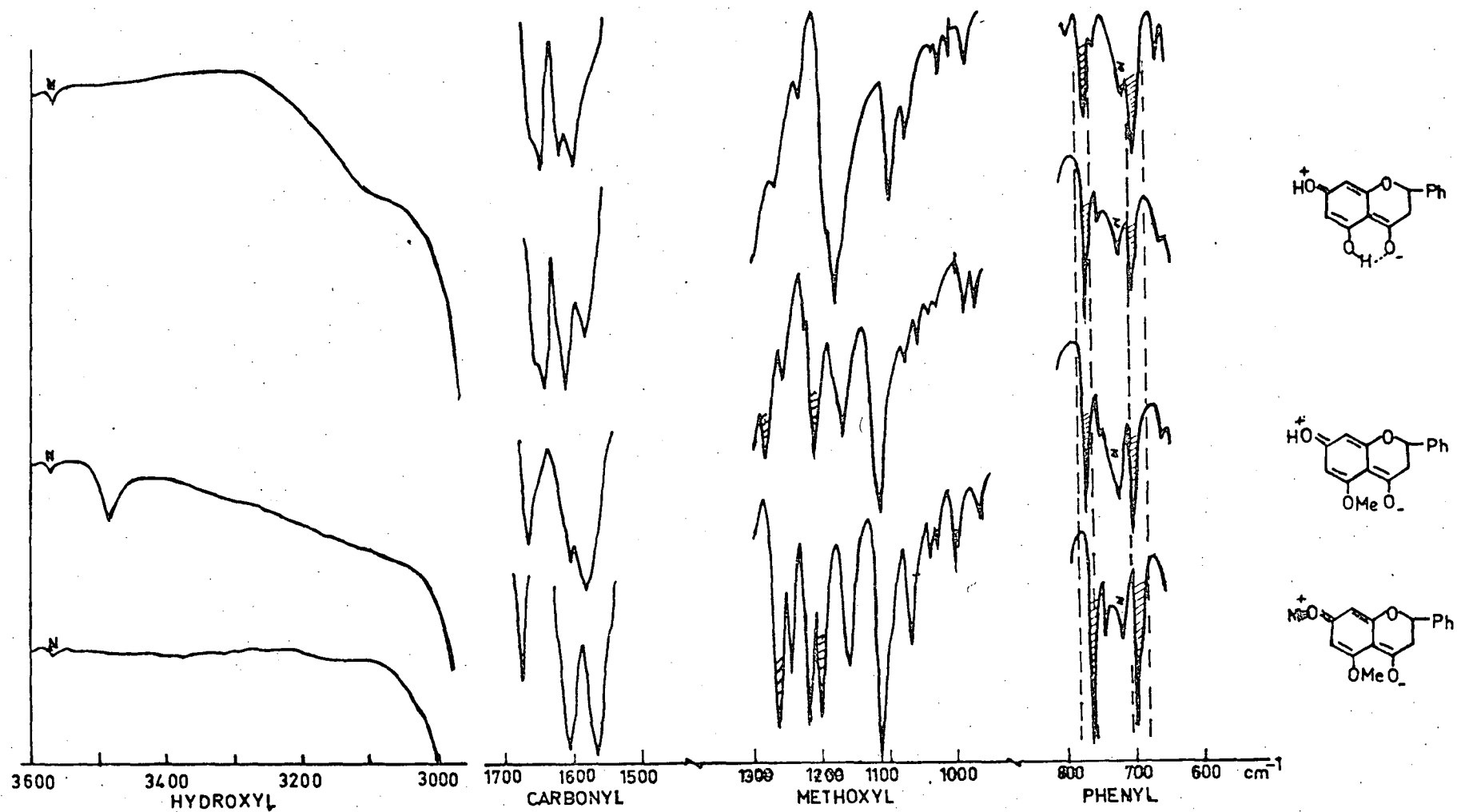
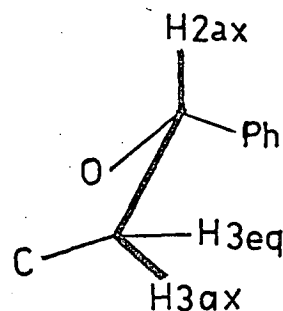
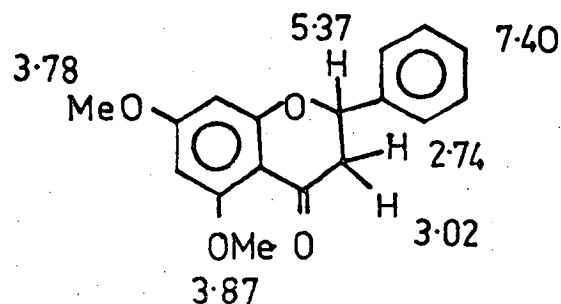
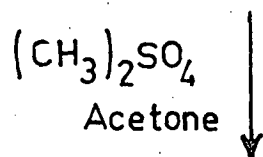
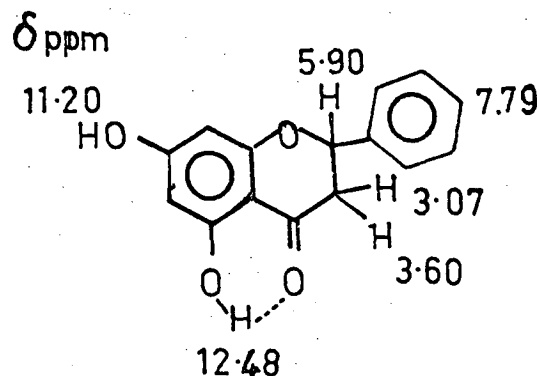


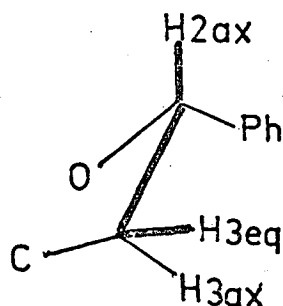
CHART 23. I.R. SPECTRAL DATA for E.sieberi FLAVANONES.

# NMR SPECTRAL DATA for *E.sieberi* FLAVANONES.

## PINOCEMBRIN

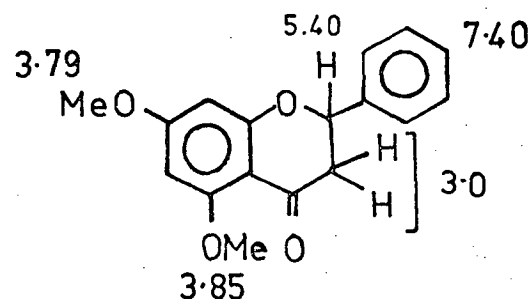
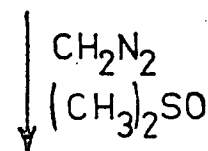
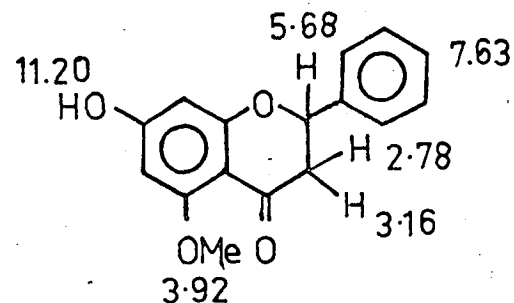


$$J \approx 13 \text{ Hz}$$



$$J \approx 3 \text{ Hz}$$

## ALPINETIN



## 5,7-DIMETHOXYFLAVANONE

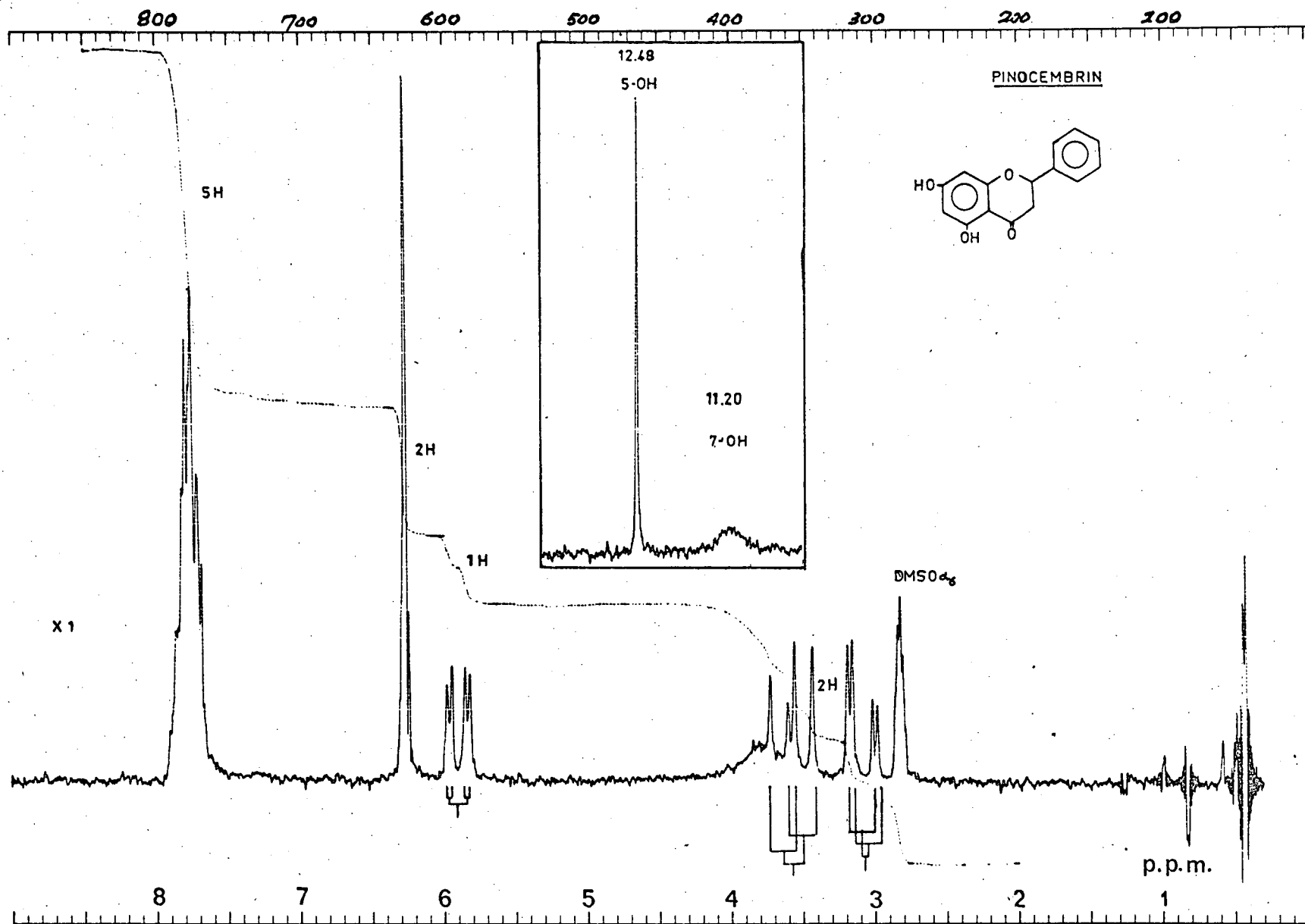


CHART 25.

N.M.R. SPECTRUM of PINOCEMBRIN.

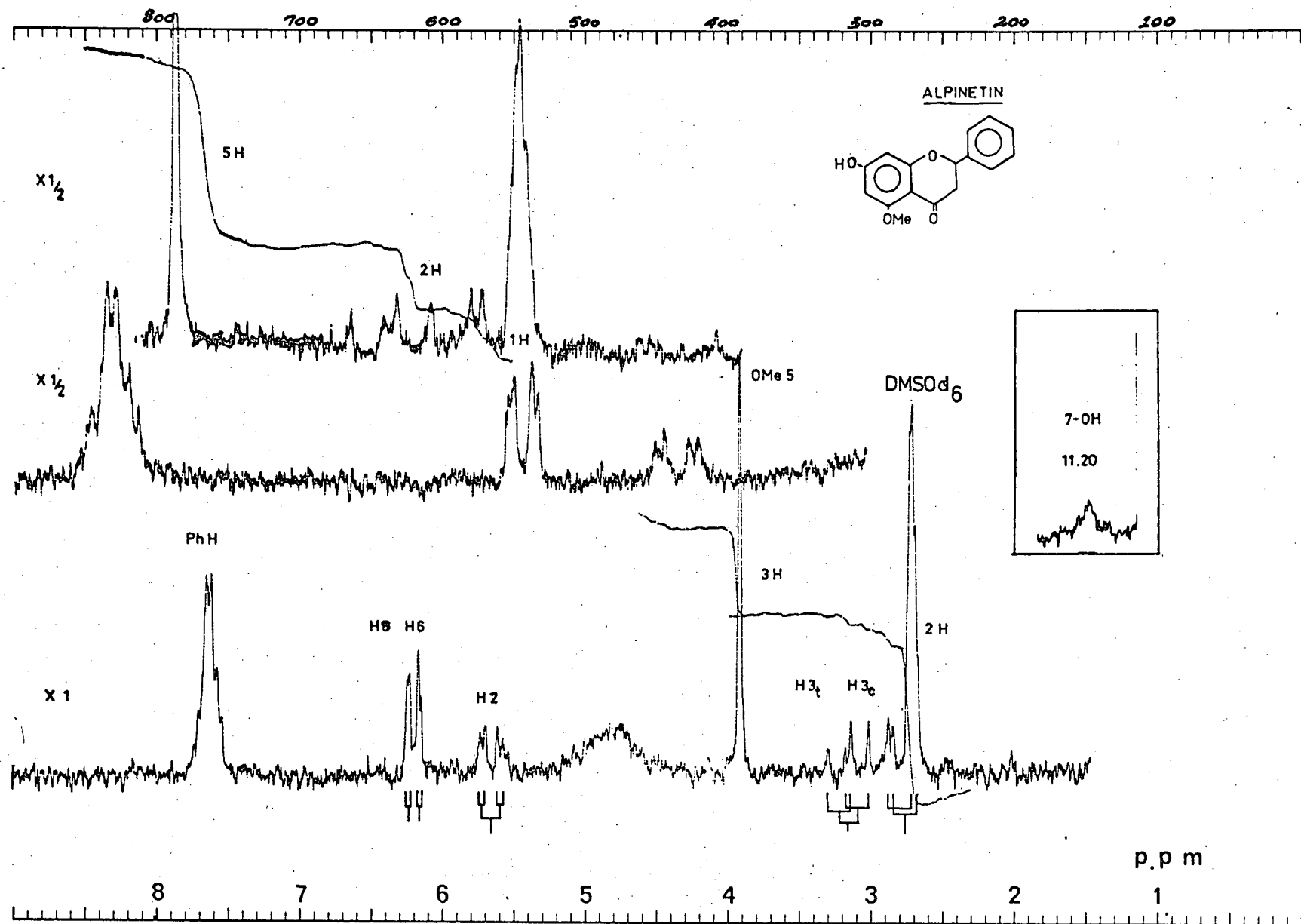


CHART 26. N.M.R. SPECTRUM of ALPINETIN.

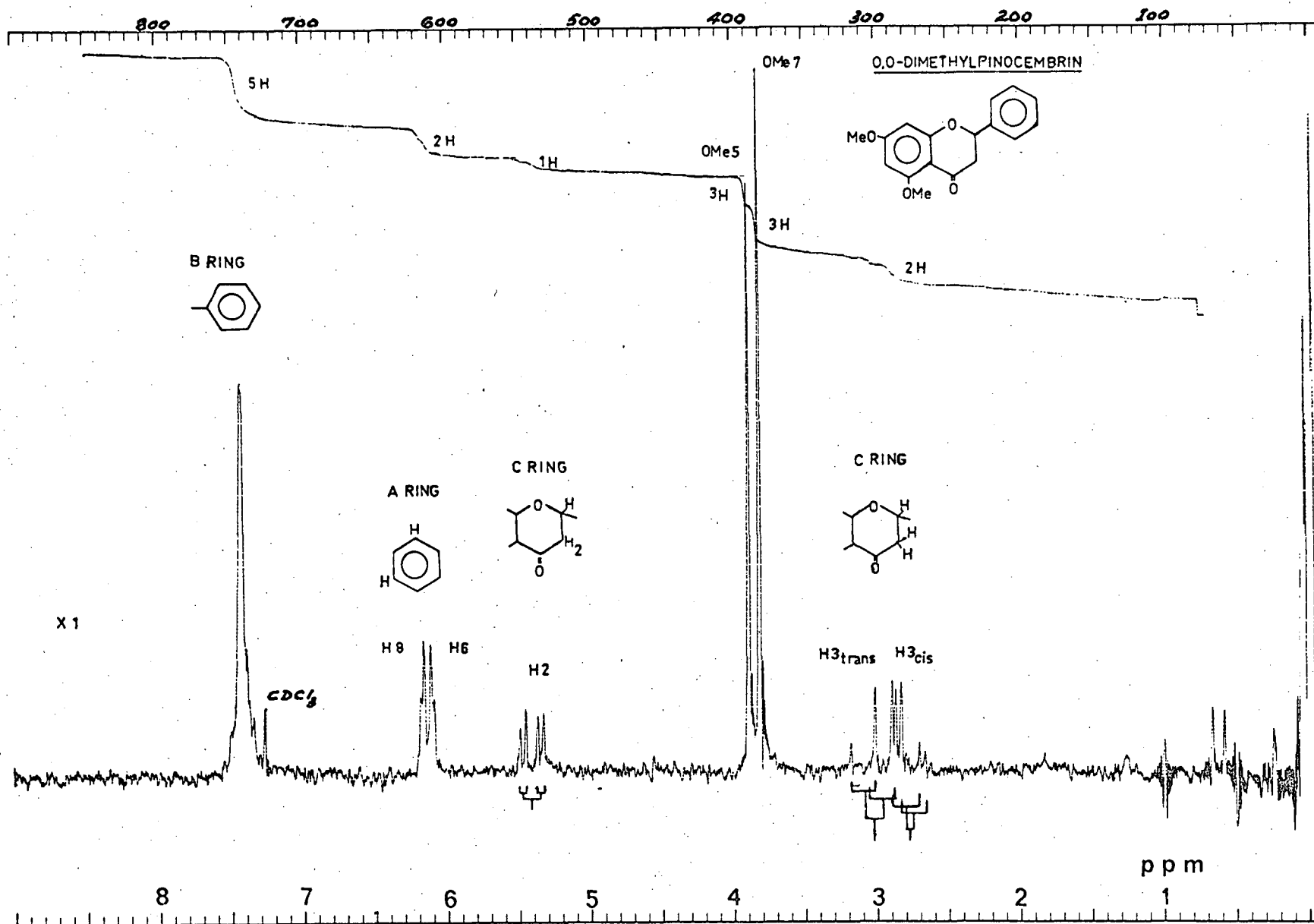


CHART 27. N.M.R. SPECTRUM of O,O-DIMETHYLPINOCEMBRIN.



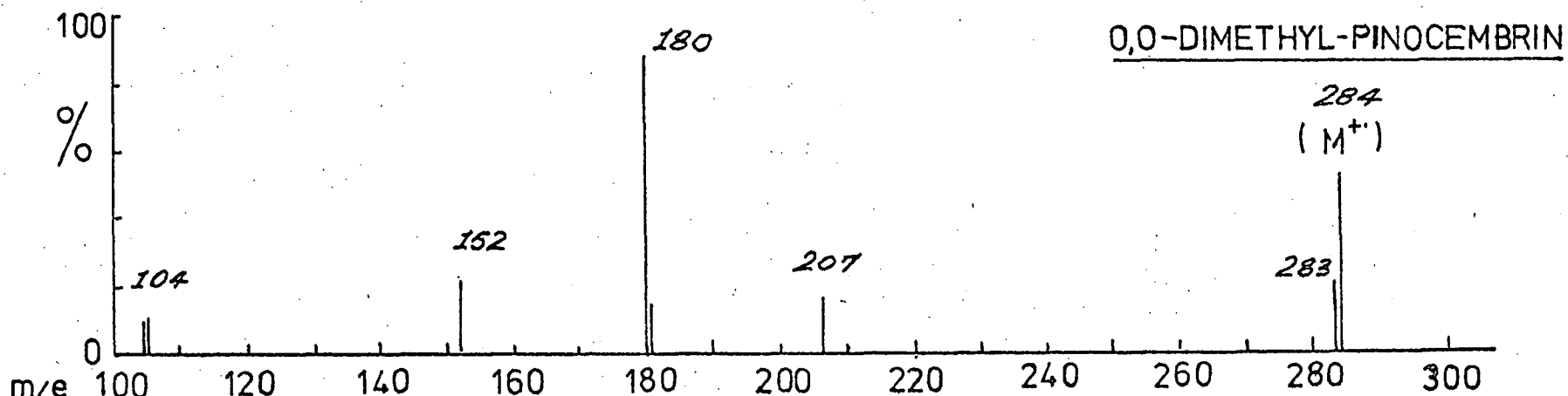
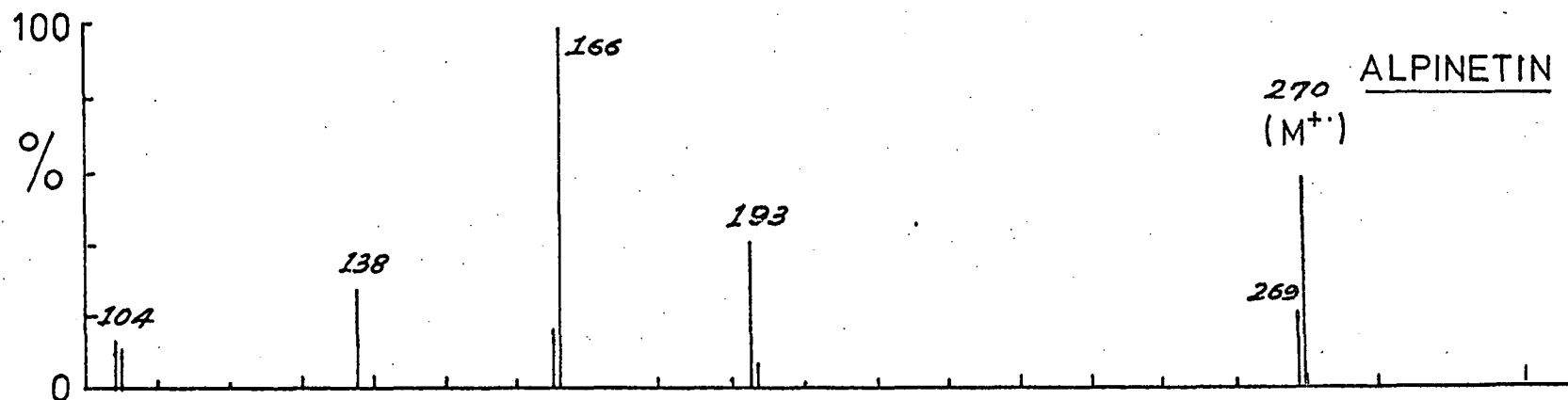
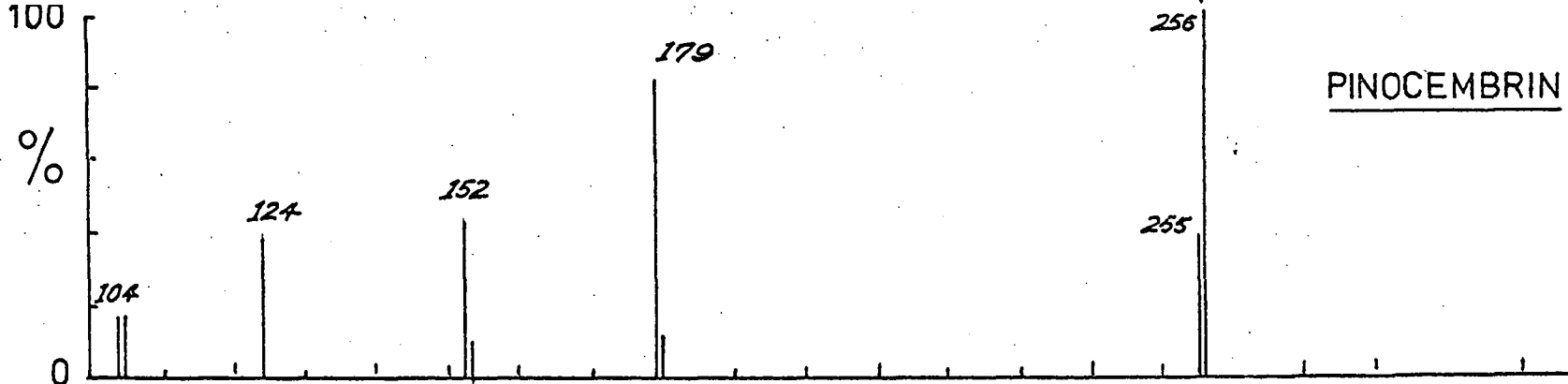


CHART 28. MASS SPECTRA of E.sieberi FLAVANONES.

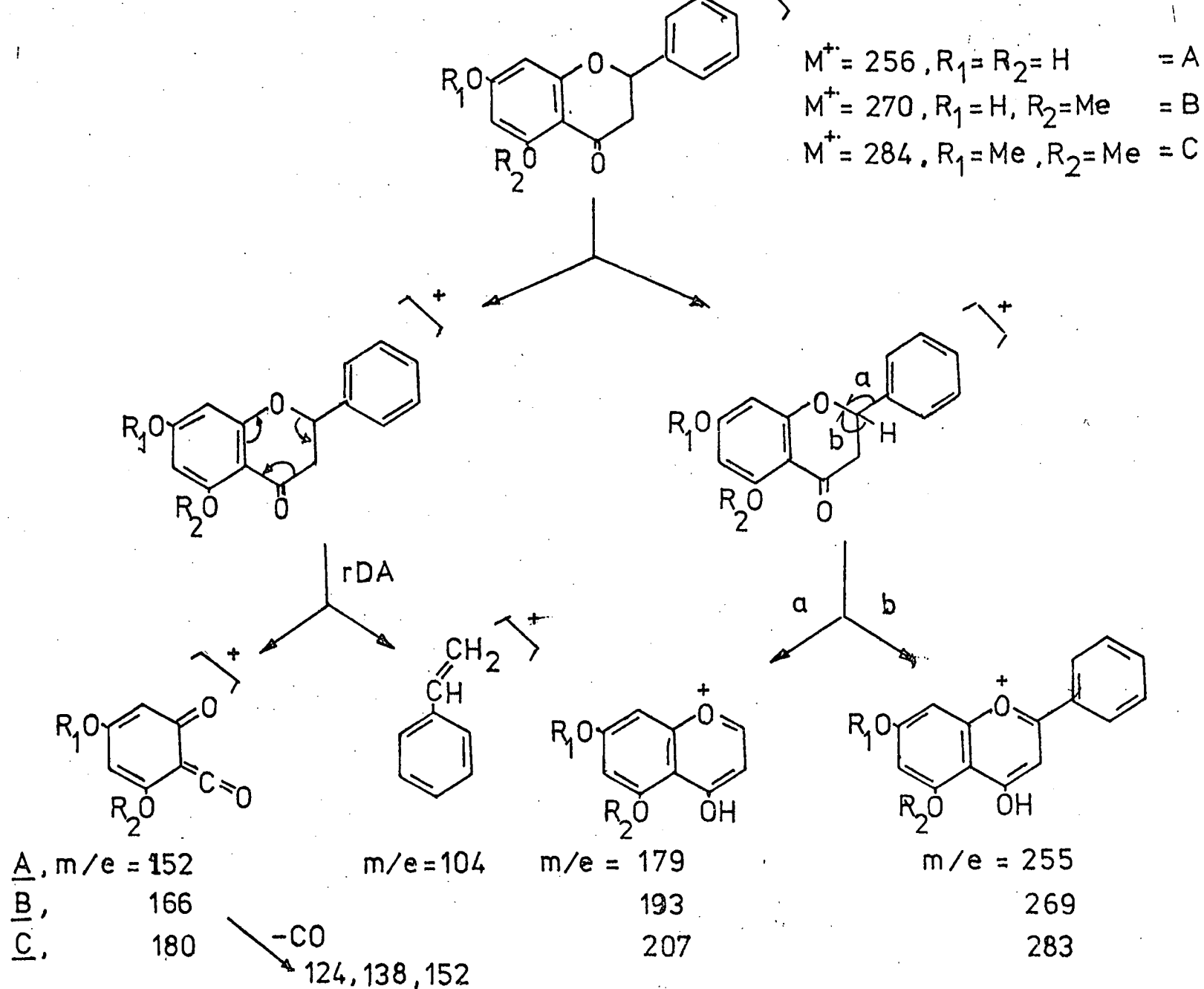
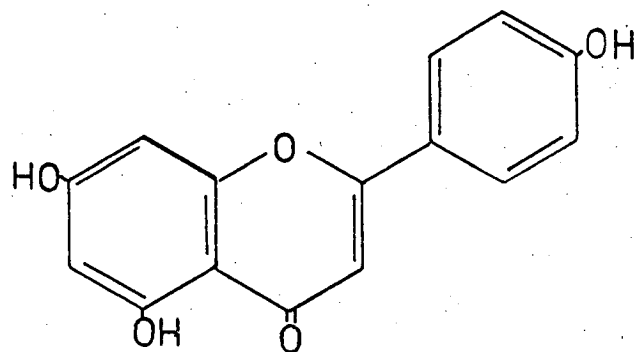


CHART 29. FRAGMENTATION of *E.sieberi* FLAVANONES.



APIGENIN

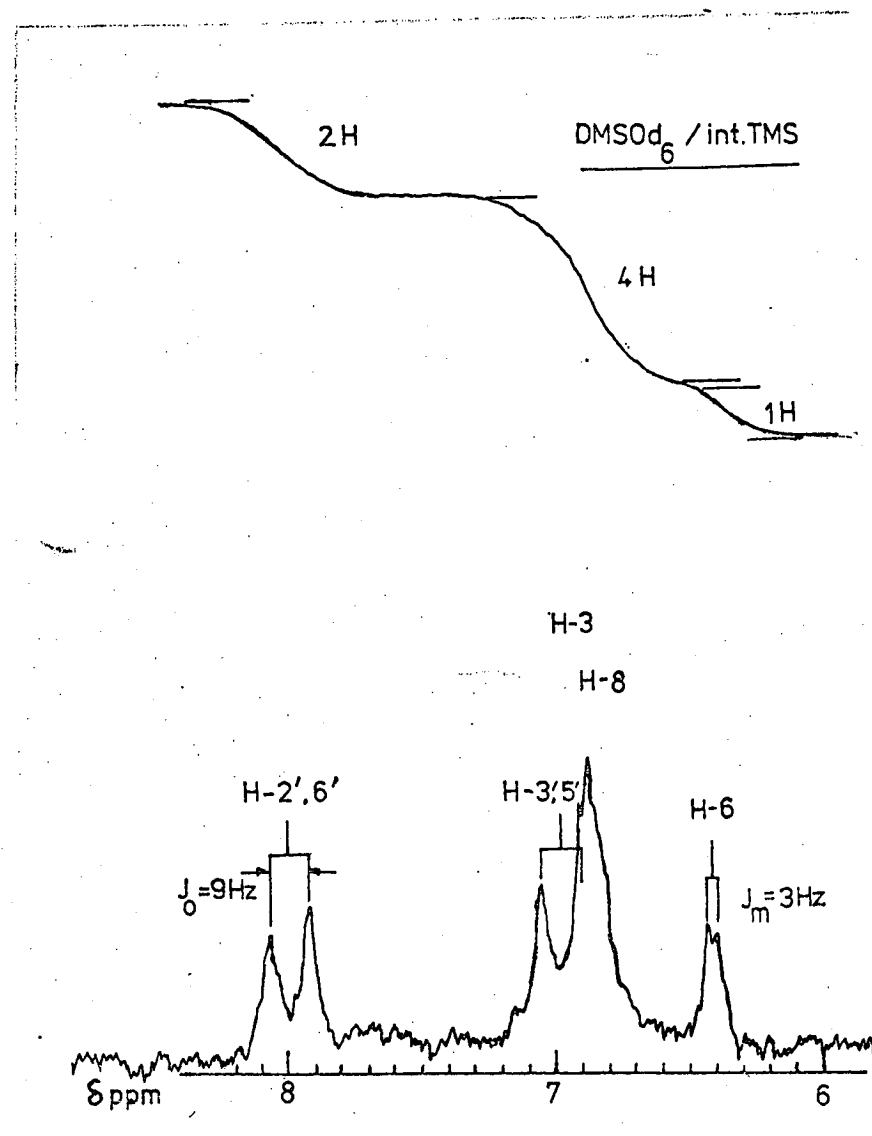
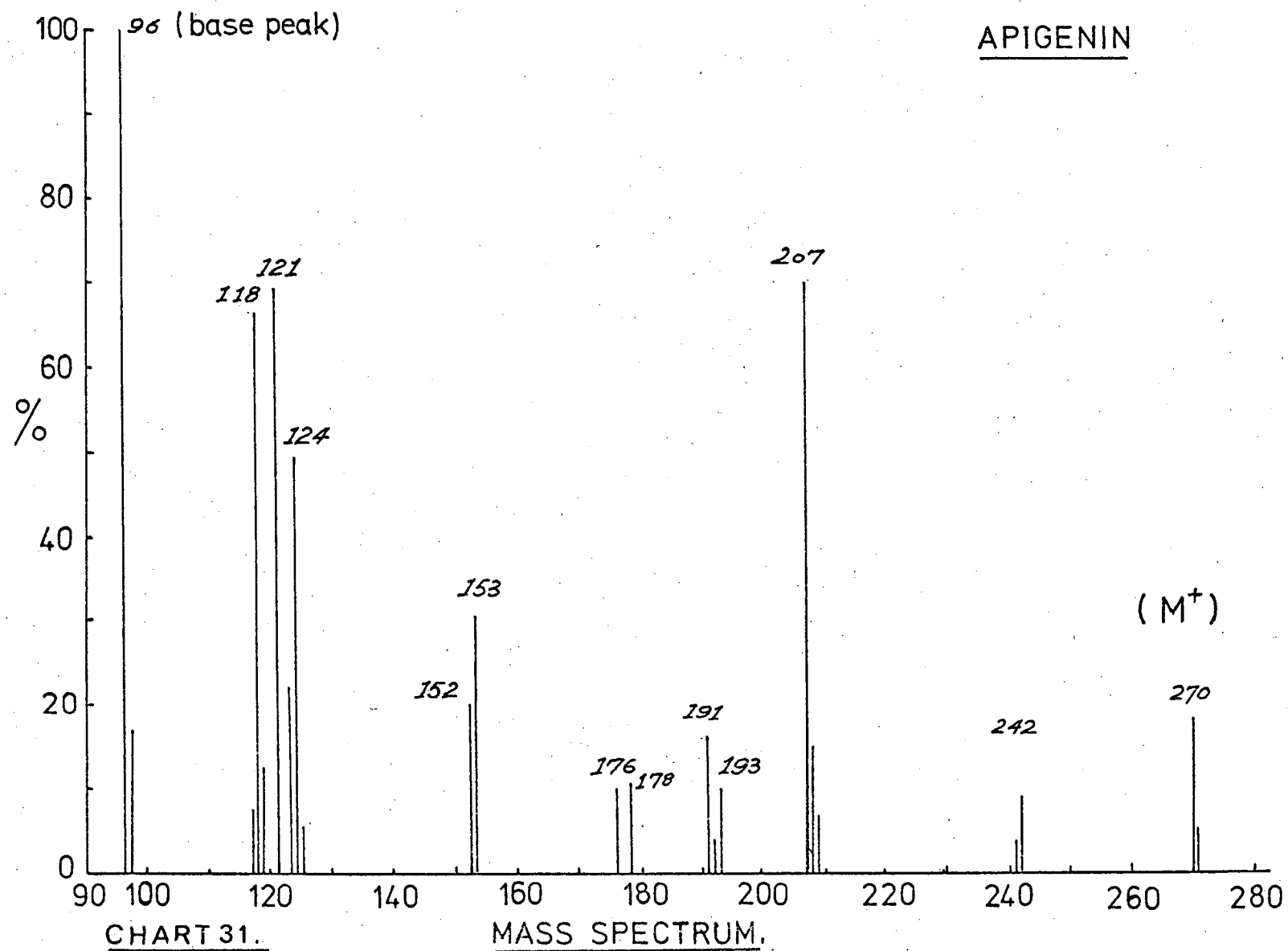


CHART 30. N.M.R. SPECTRUM of APIGENIN.

APIGENIN



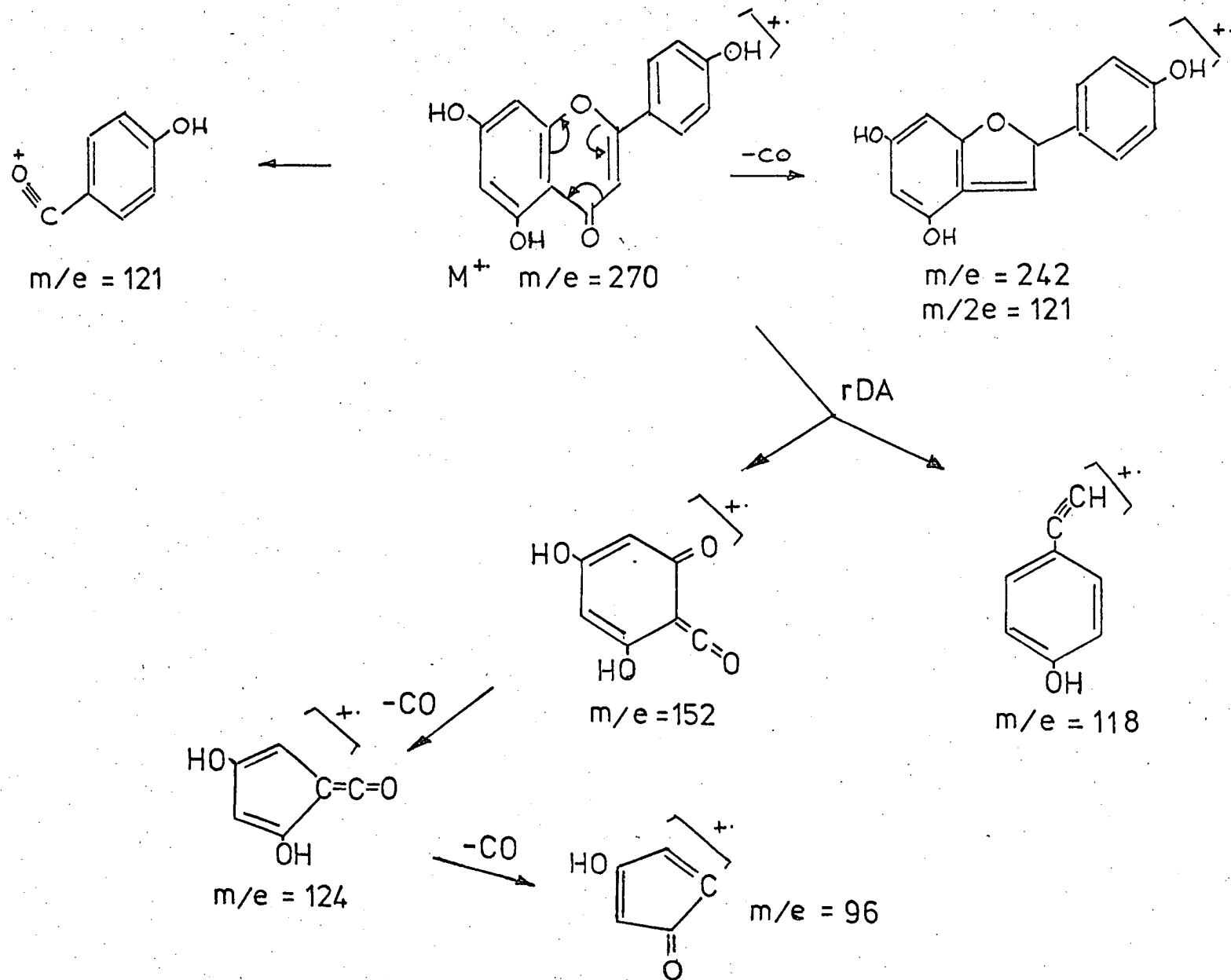


CHART 32. MASS FRAGMENTATION of APIGENIN.

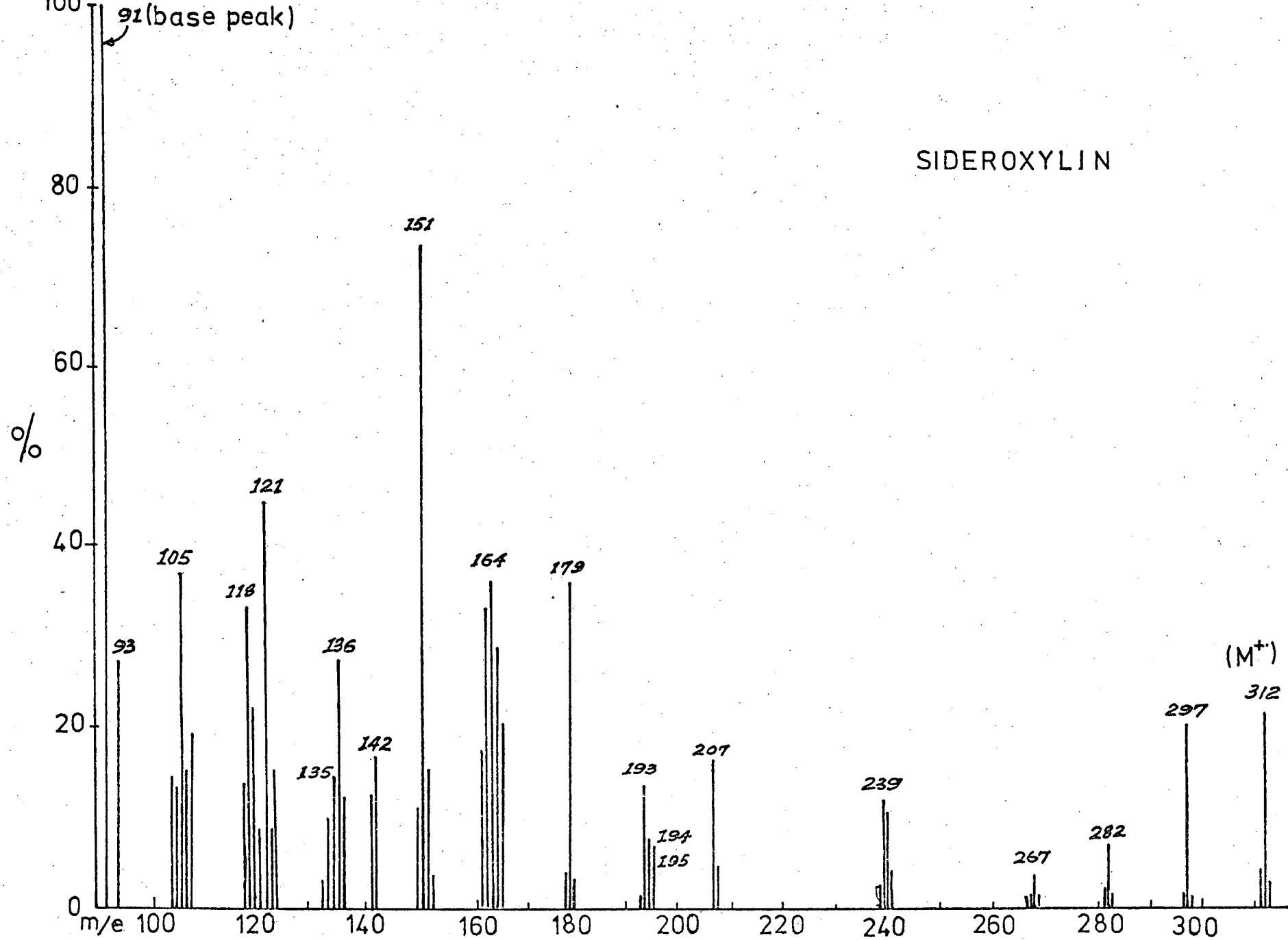


CHART 33.

MASS SPECTRUM.

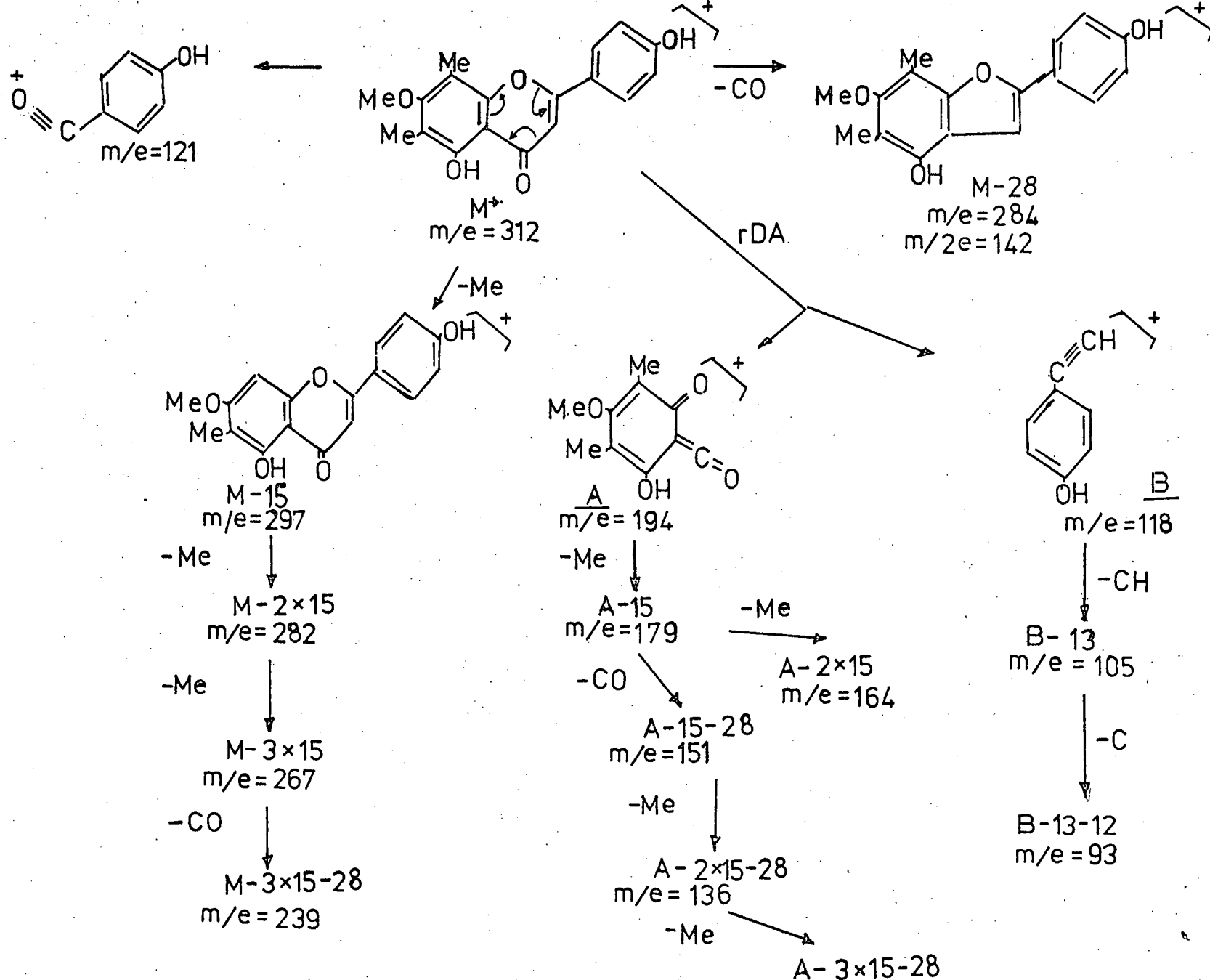
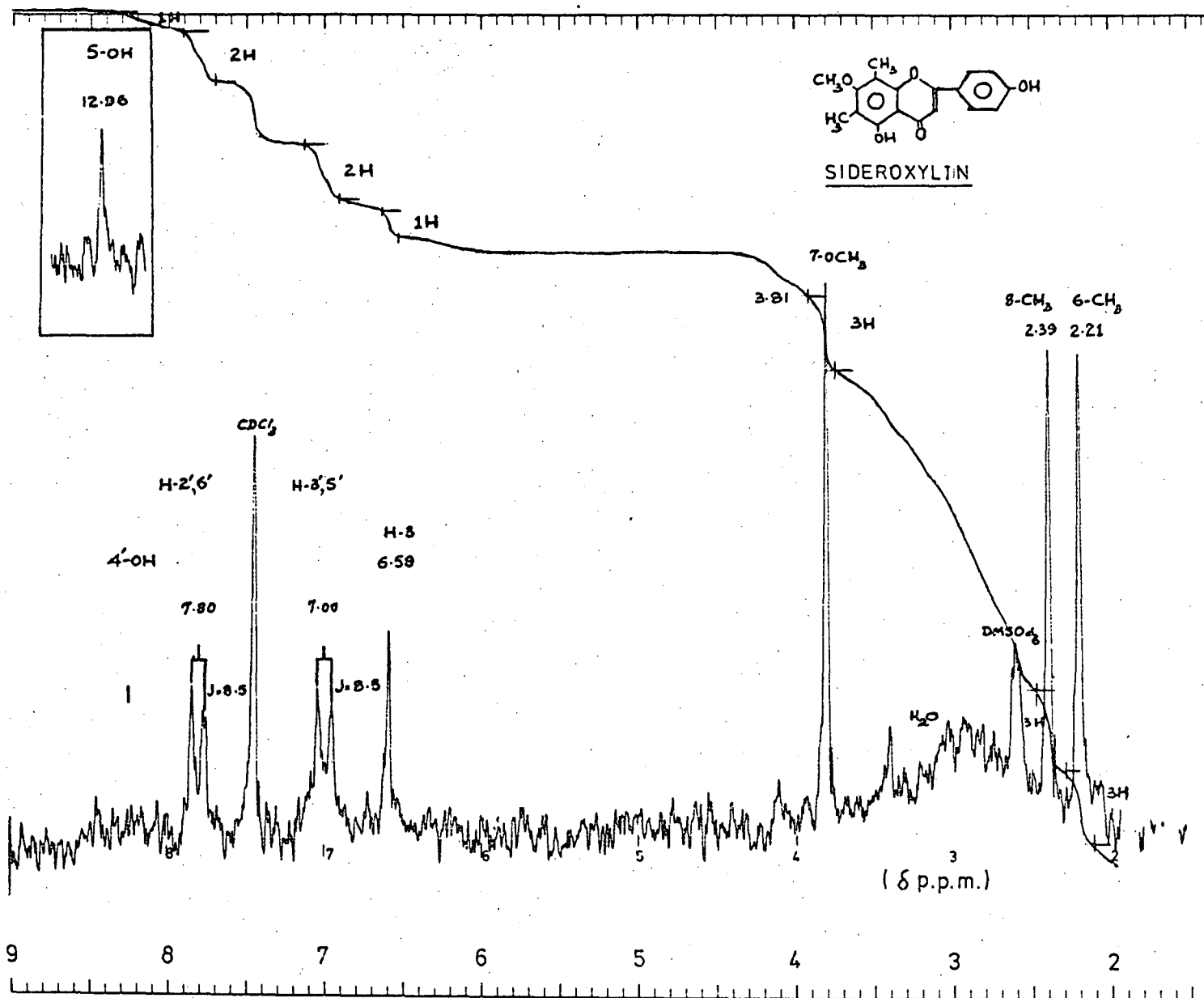
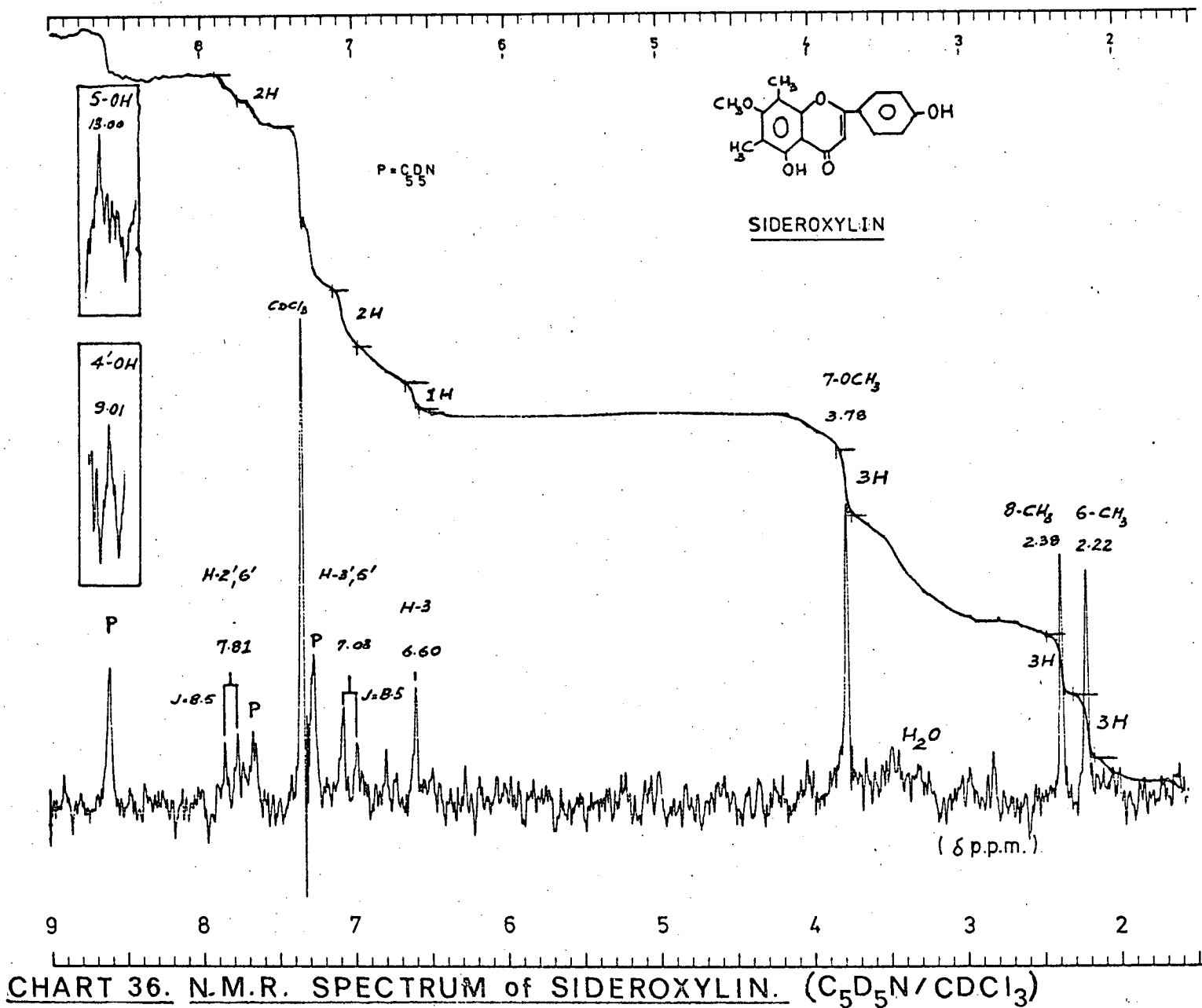


CHART 34. MASS FRAGMENTATION of SIDEROXYLIN.



**CHART 35. N.M.R. SPECTRUM of SIDEROXYLIN. (DMSO-d<sub>6</sub>/CDCl<sub>3</sub>)**





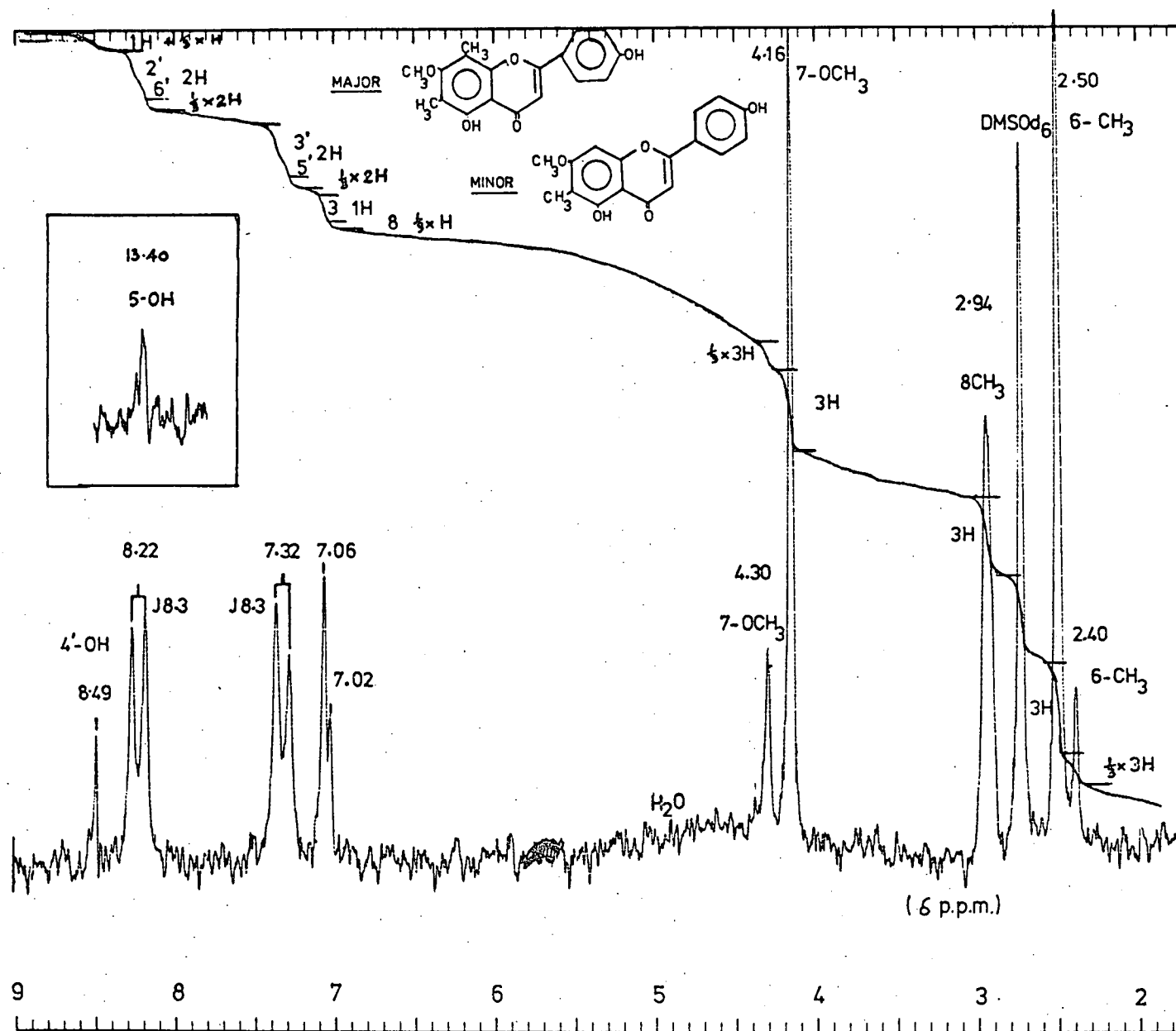


CHART 37. N.M.R. SPECTRUM of MIXTURE of SIDEROXYLIN + C-METHYLFLAVONE.

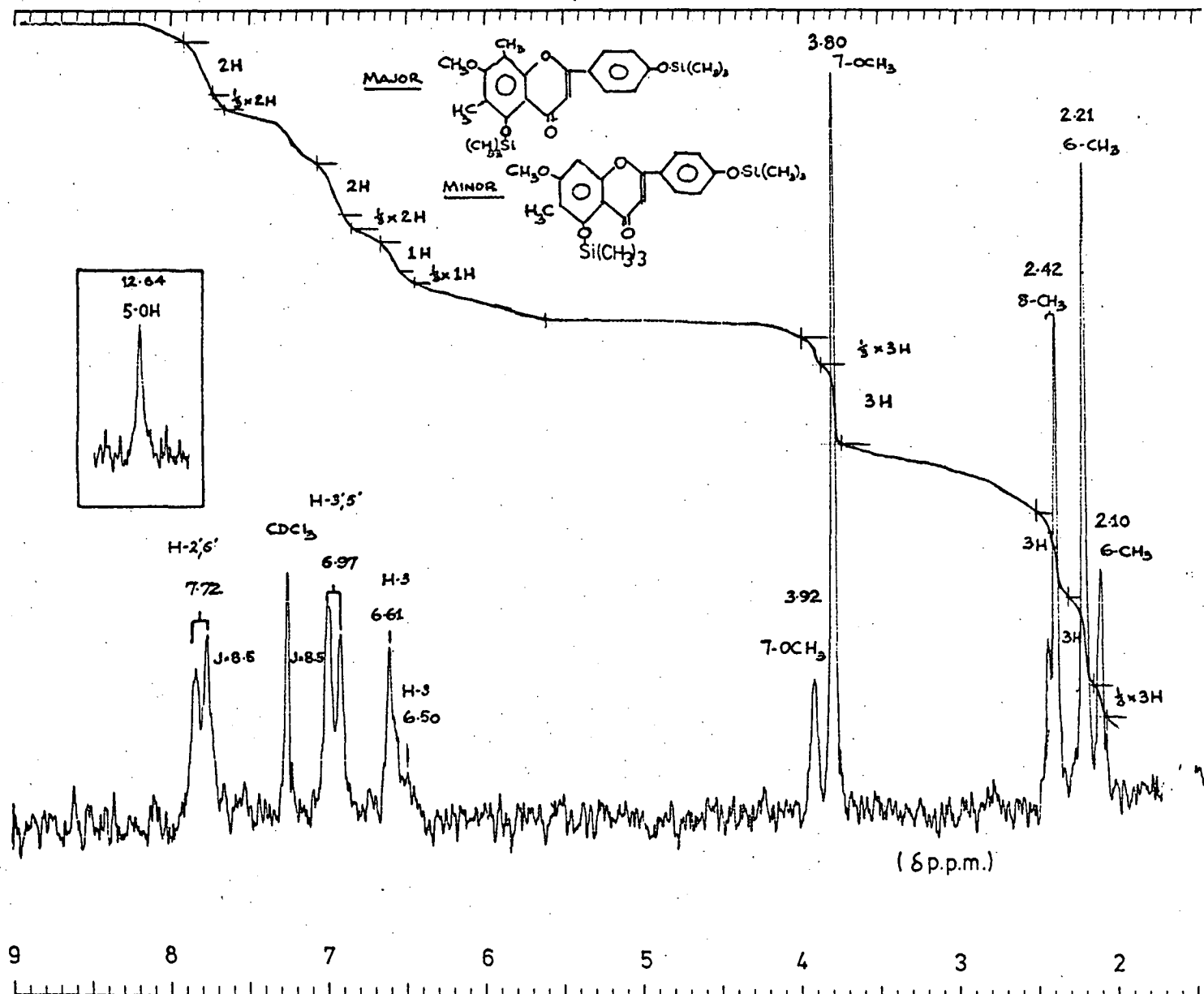


CHART 38. N.M.R. SPECTRUM of TMS ETHER of SIDEROXYLIN + C-METHYLFLAVONE.

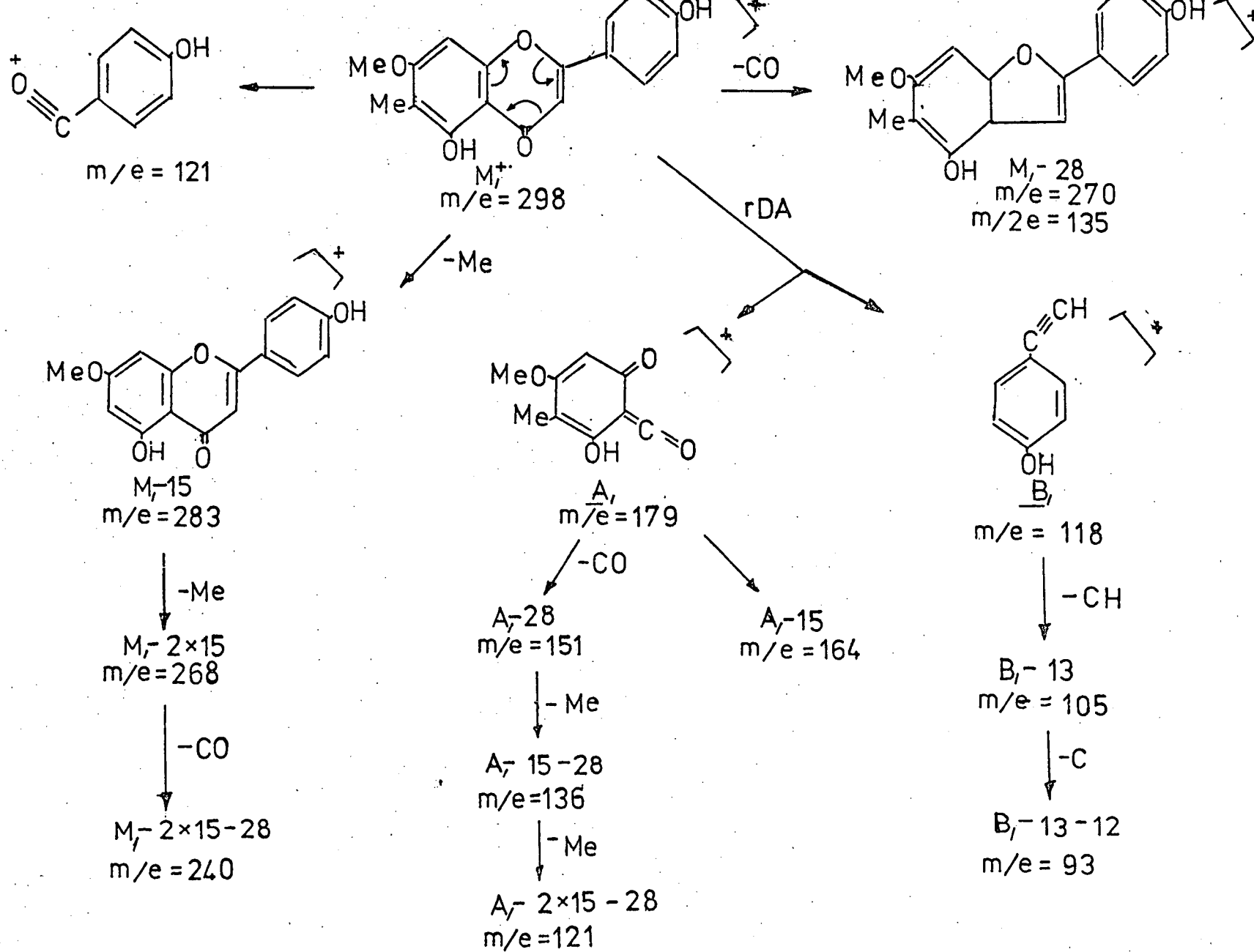


CHART 39. MASS FRAGMENTATION of SECOND C-METHYLFLAVONE.

# SIDEROXYLIN MIXTURE

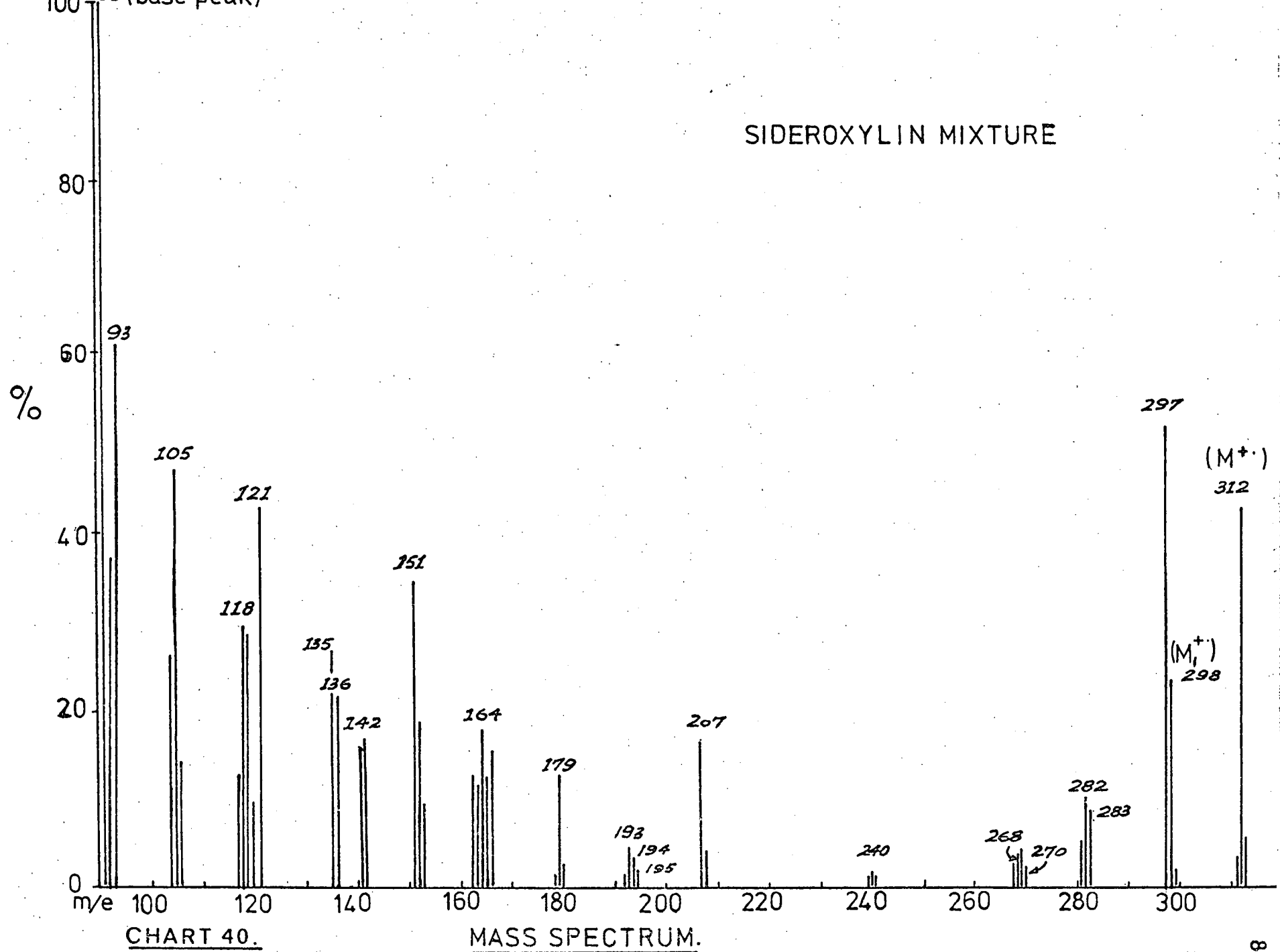
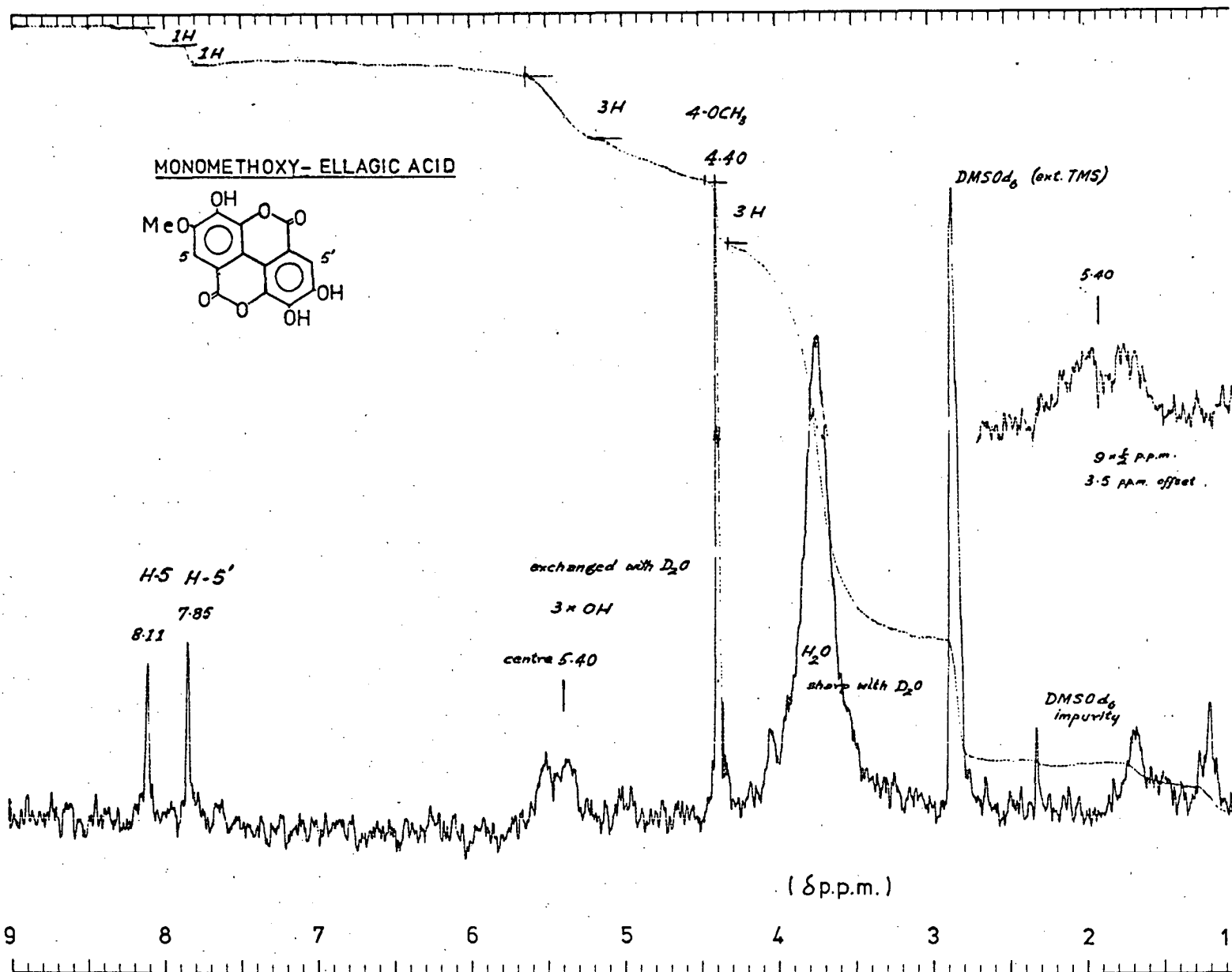
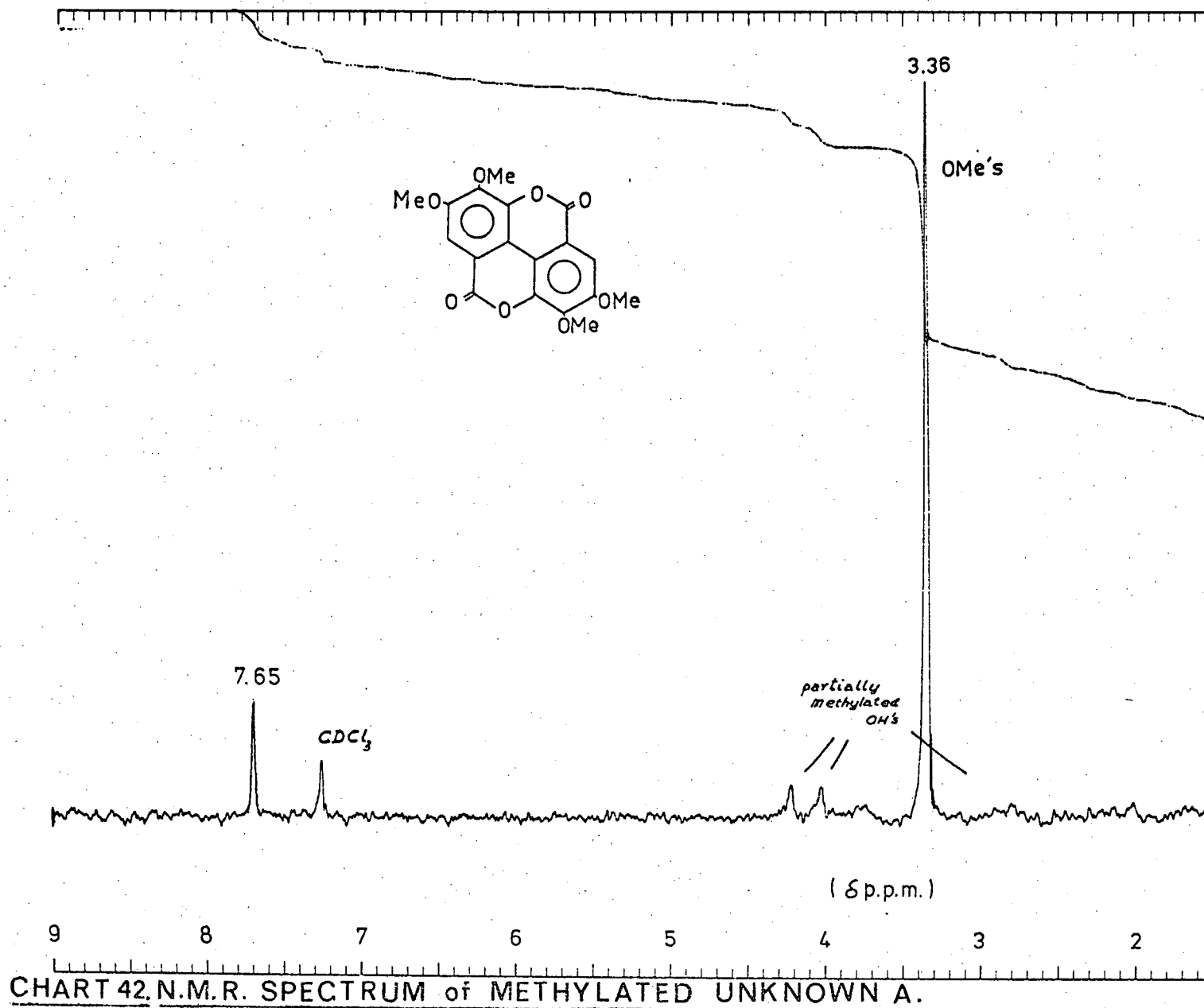


CHART 40.

MASS SPECTRUM.



**CHART 41. N.M.R. SPECTRUM of MONOMETHOXY- ELLAGIC ACID.**



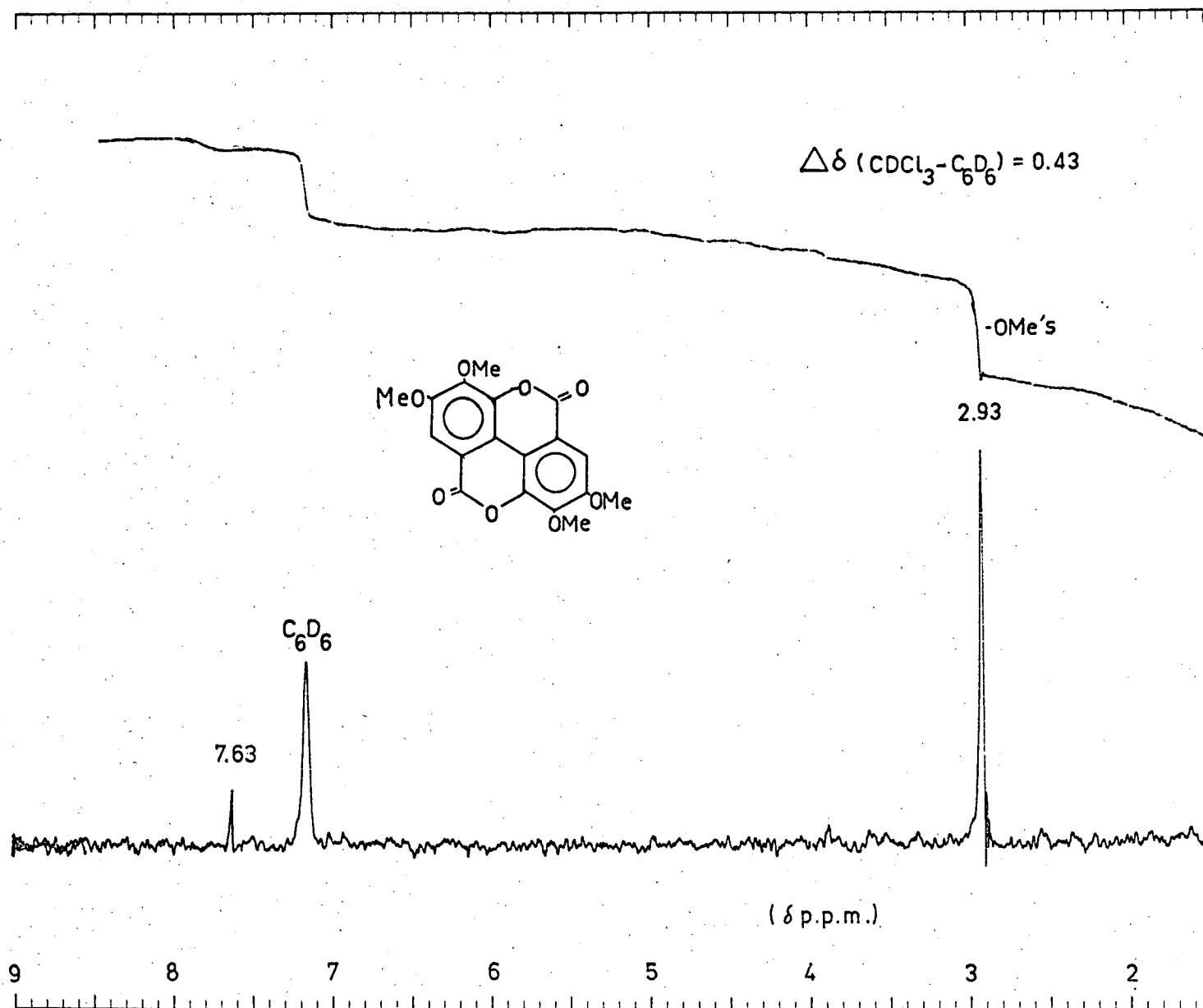


CHART 43. N.M.R. SPECTRUM of UNKNOWN A with BENZENE SHIFT.



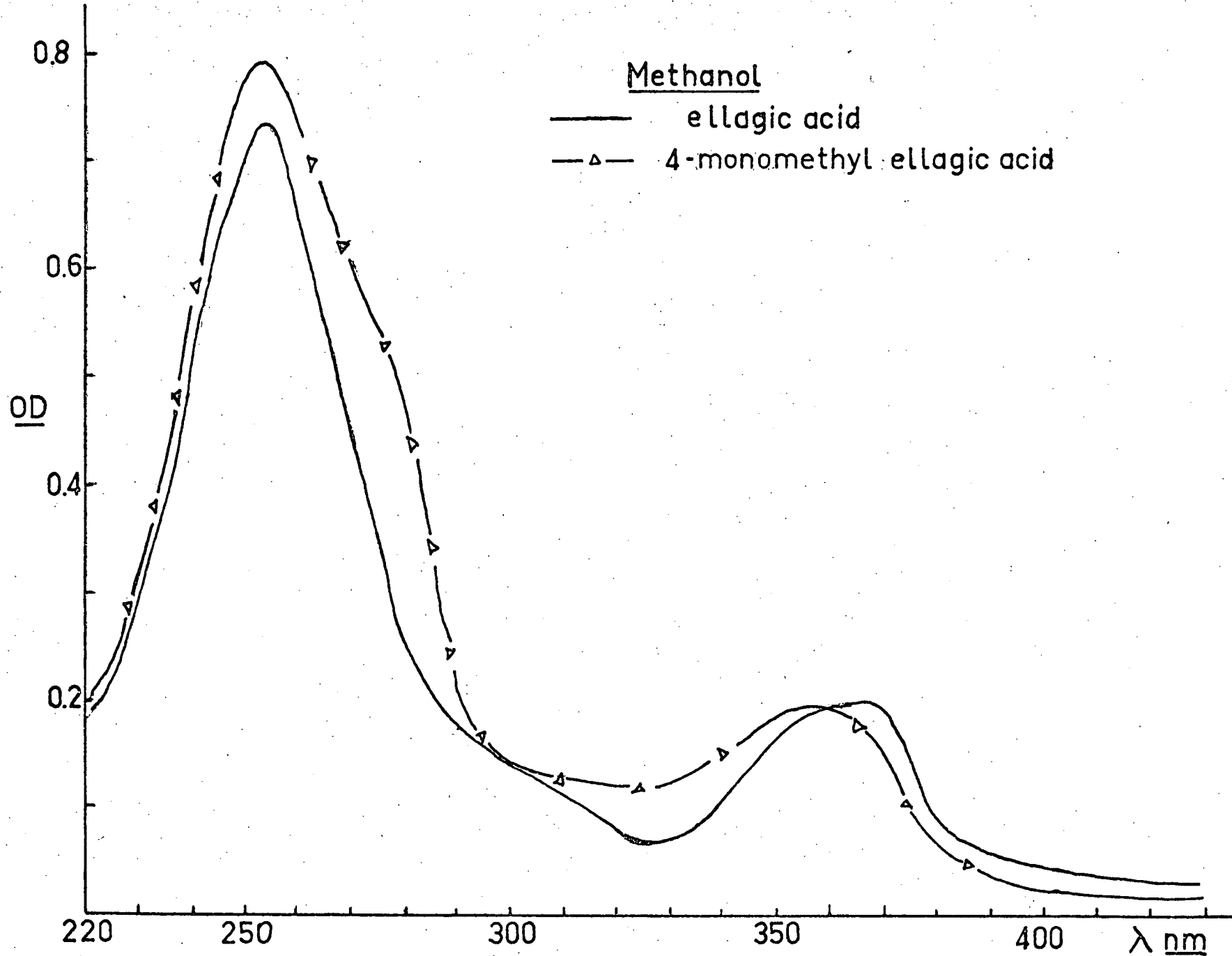


CHART 44. U.V. SPECTRA of UNKNOWN A and ELLAGIC ACID.

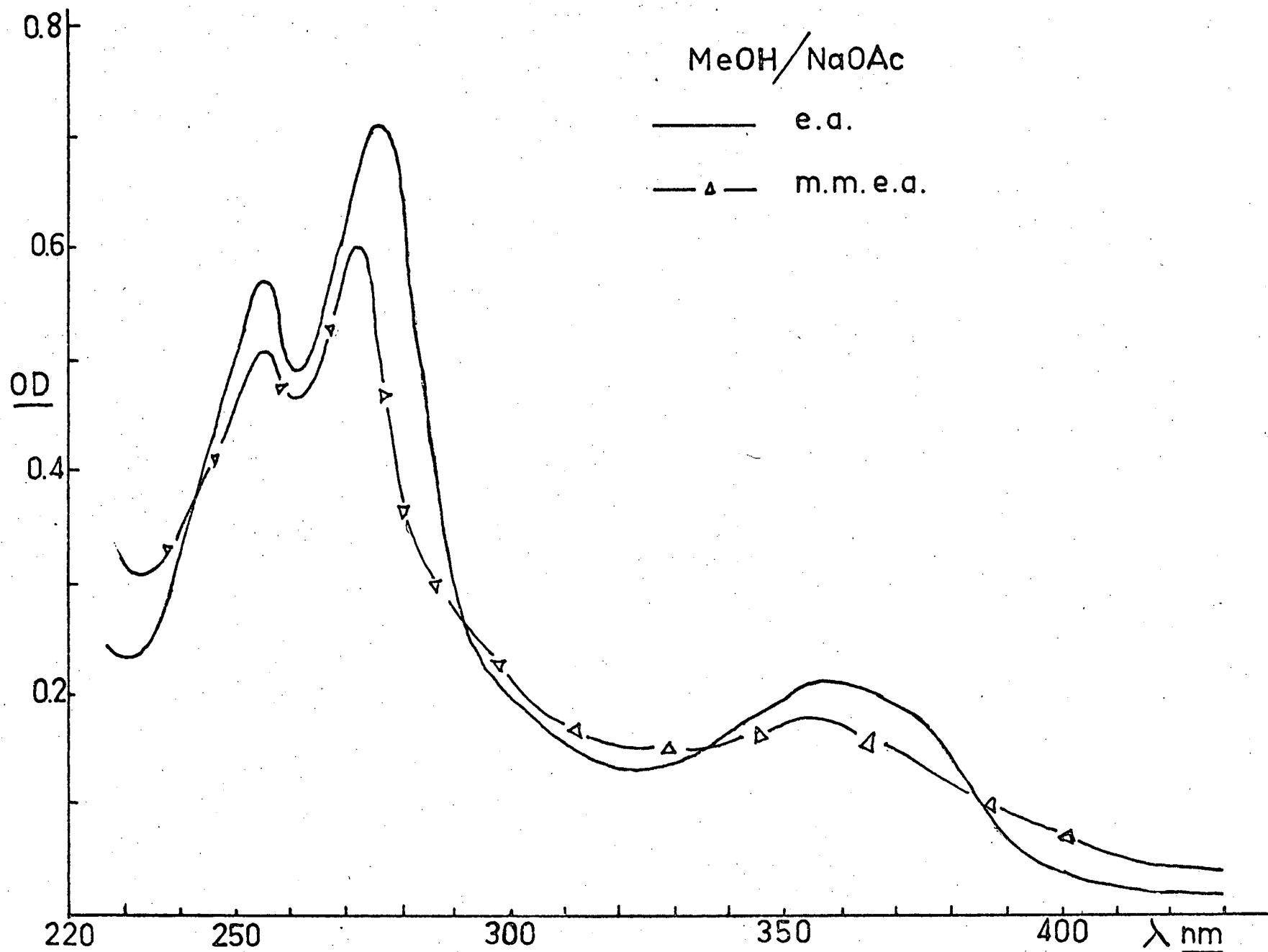


CHART 45. U.V. SPECTRA of UNKNOWN A and ELLAGIC ACID. (NaOAc)

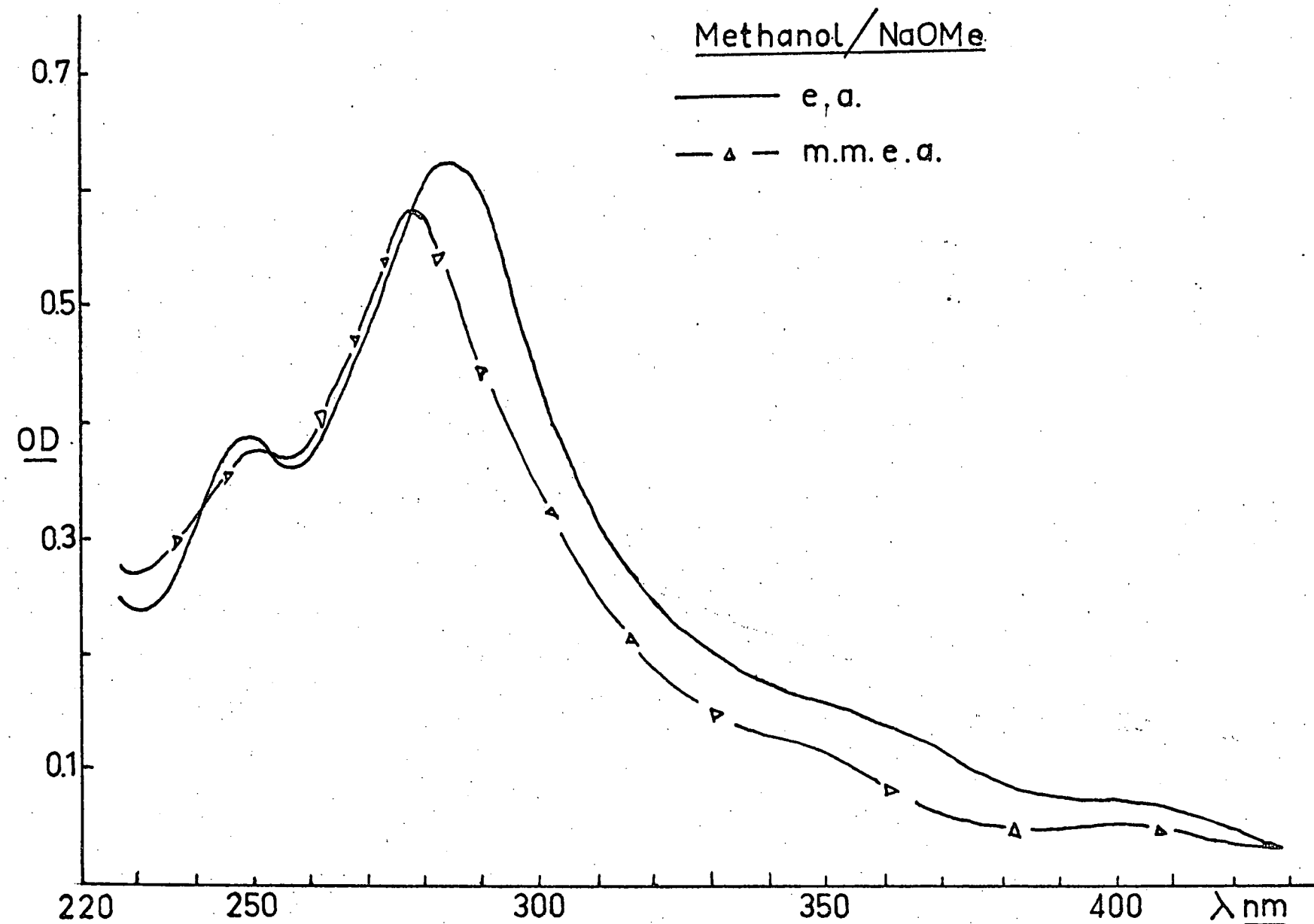


CHART 46. U.V. SPECTRA of UNKNOWN A and ELLAGIC ACID. (NaOMe)

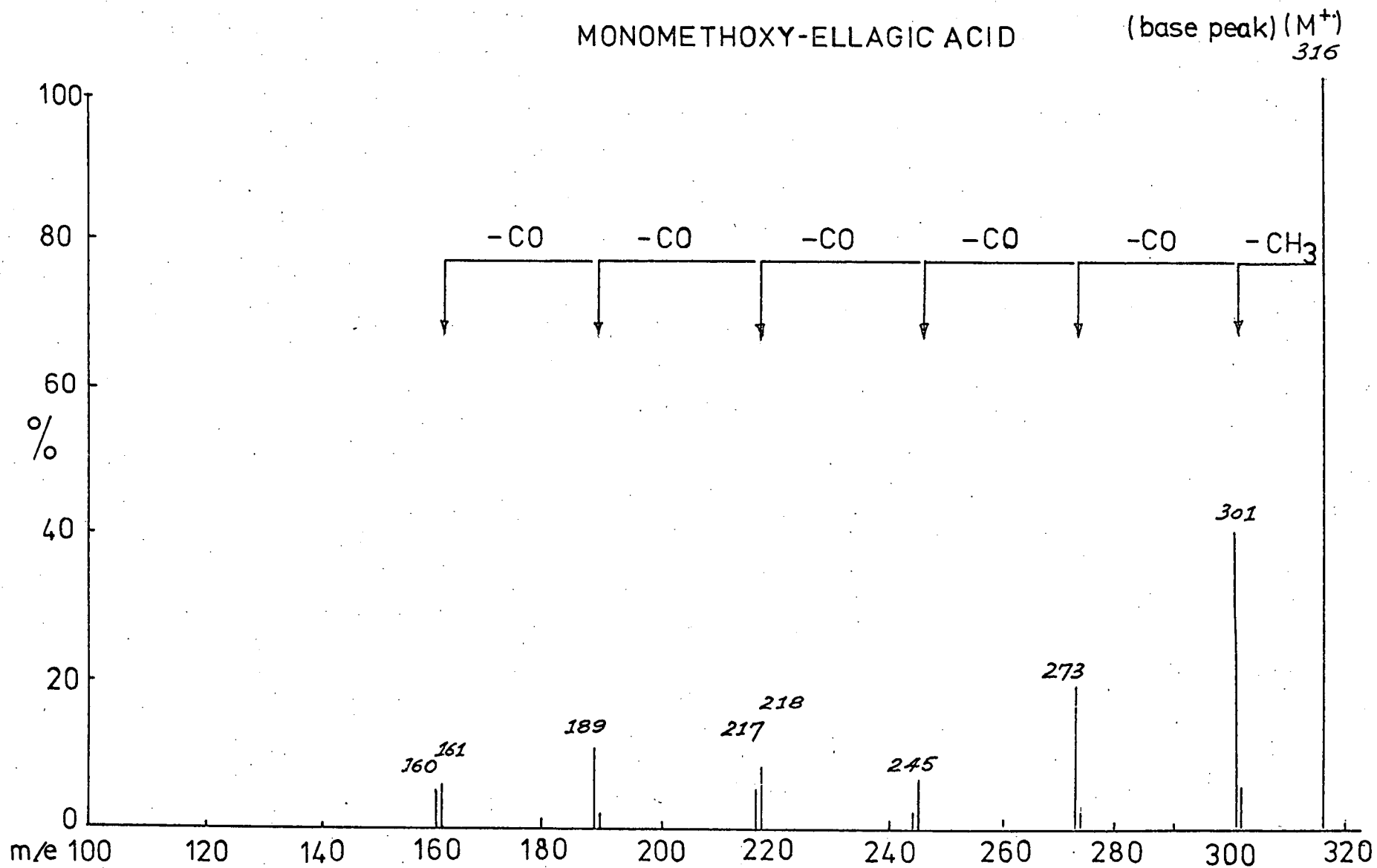
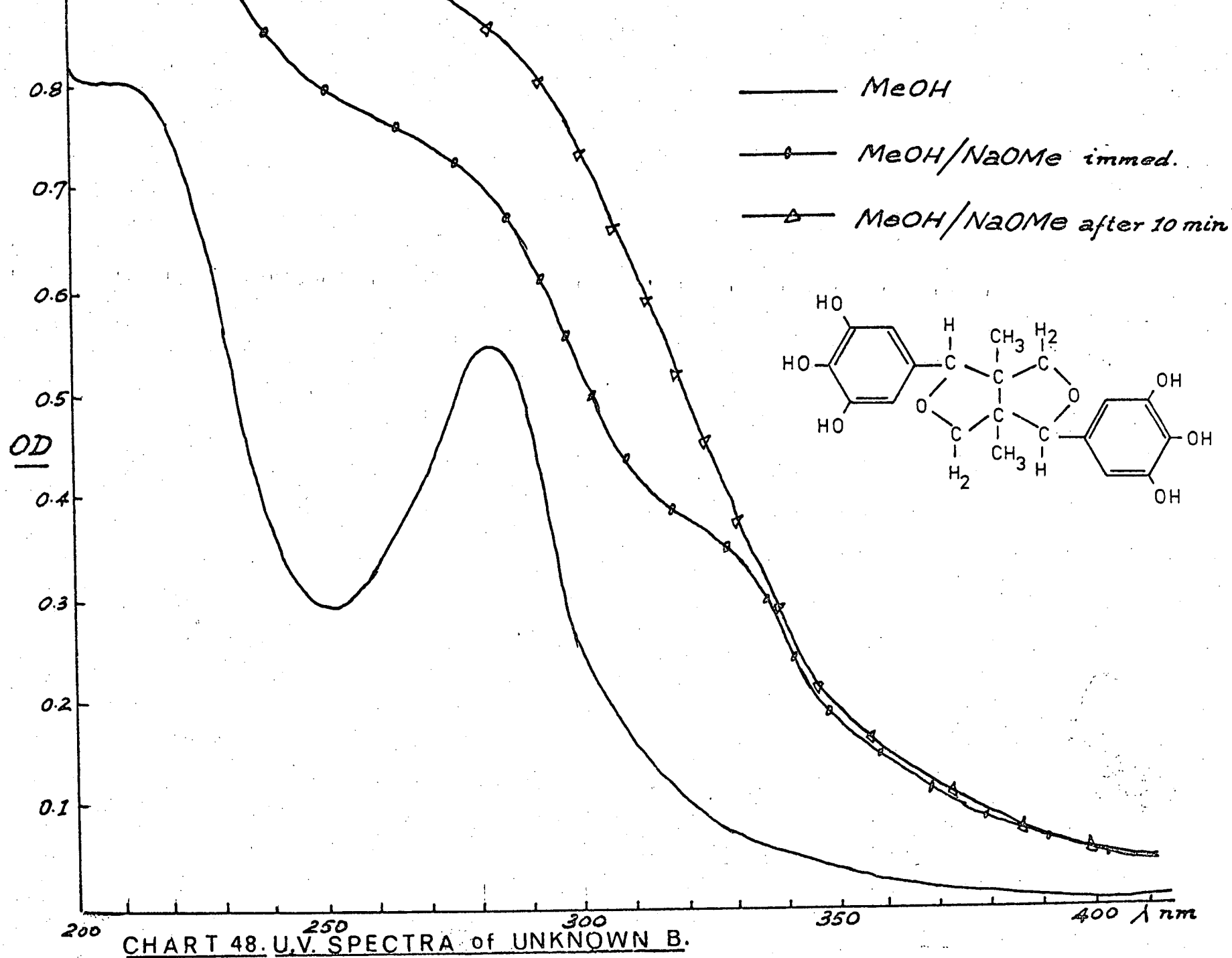


CHART 47. MASS SPECTRUM and FRAGMENTATION of ELLAGIC ACID ETHER.



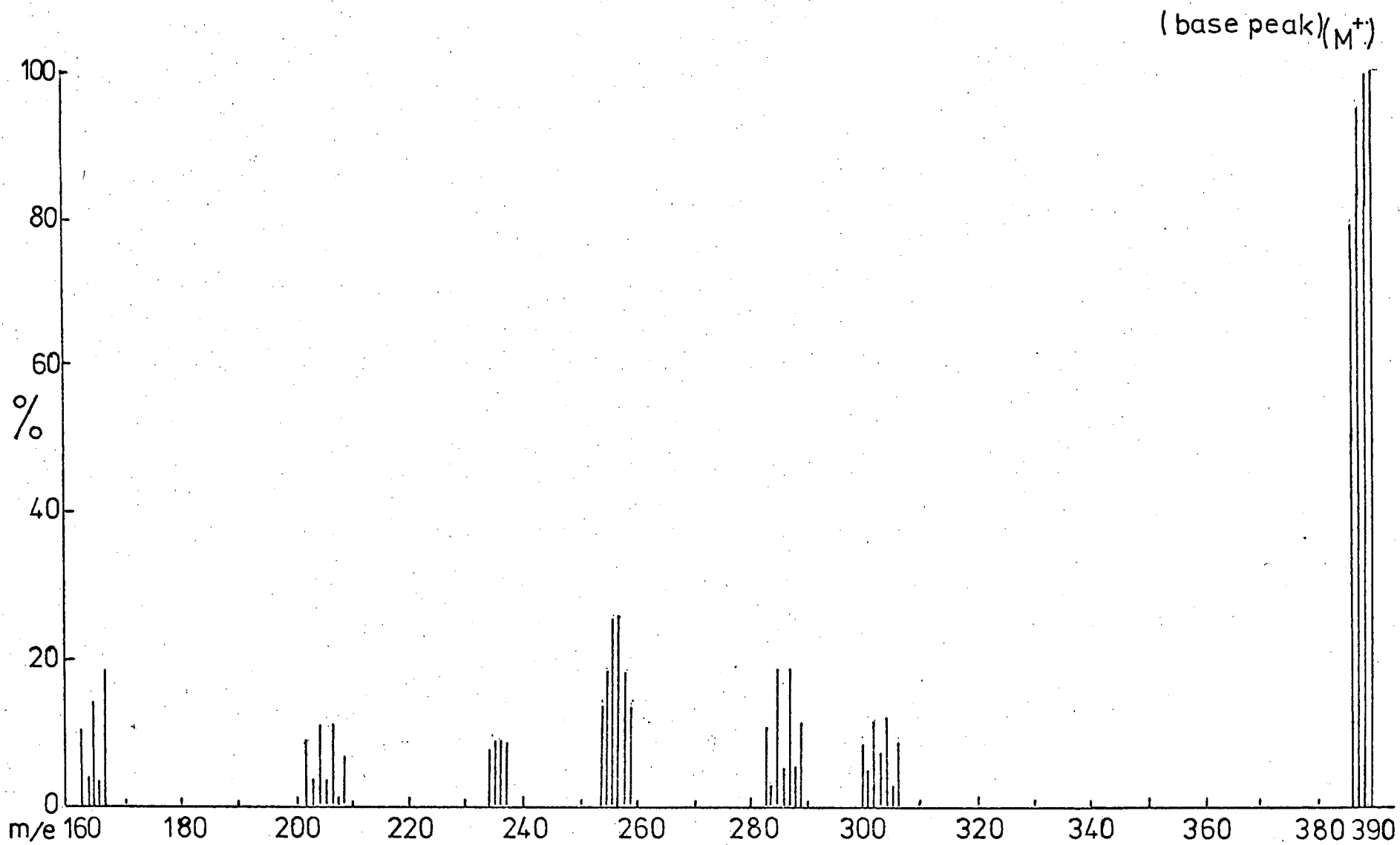


CHART 49.

MASS SPECTRUM of UNKNOWN B.

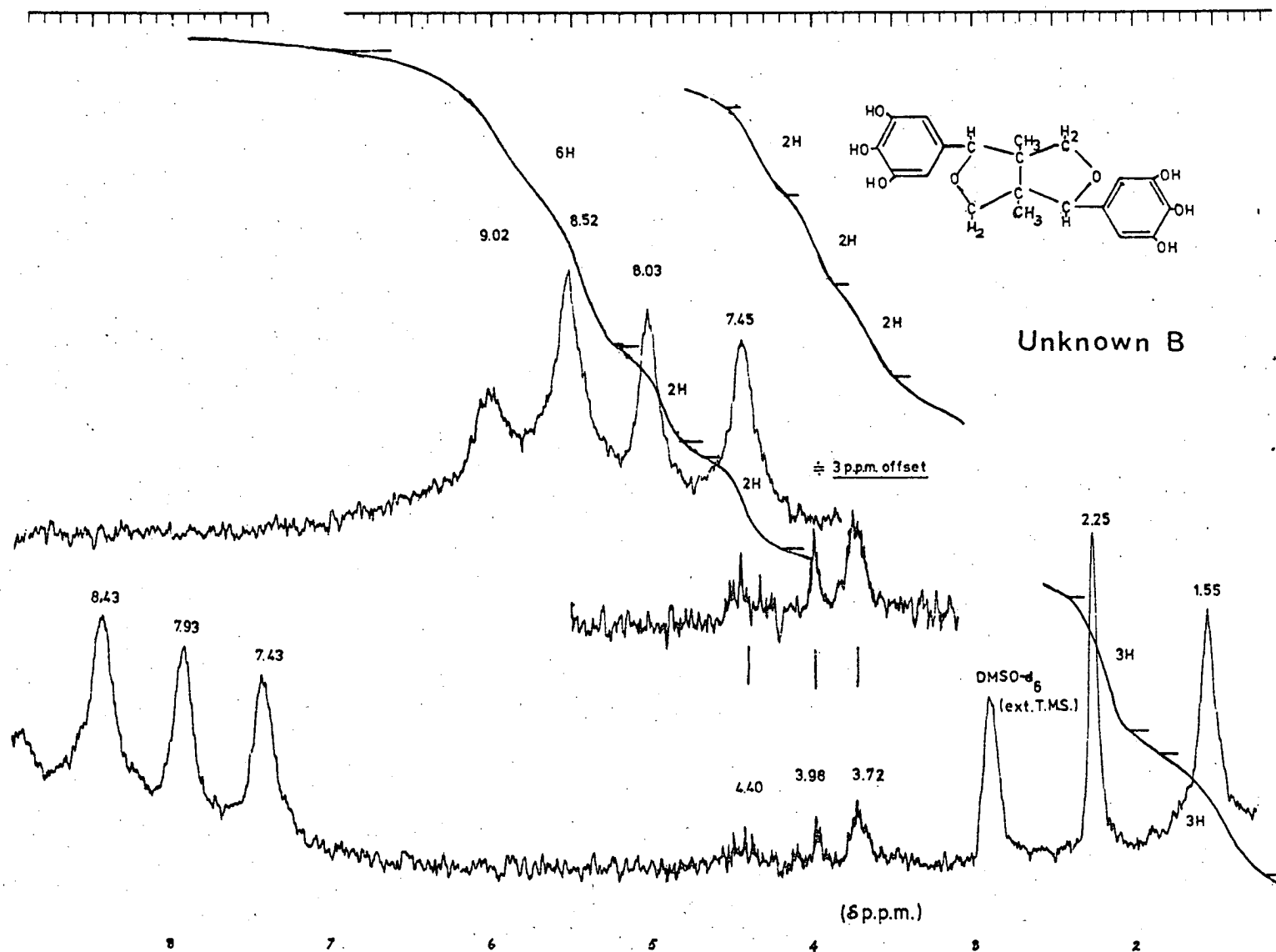


CHART 50. N.M.R. SPECTRUM of UNKNOWN B.

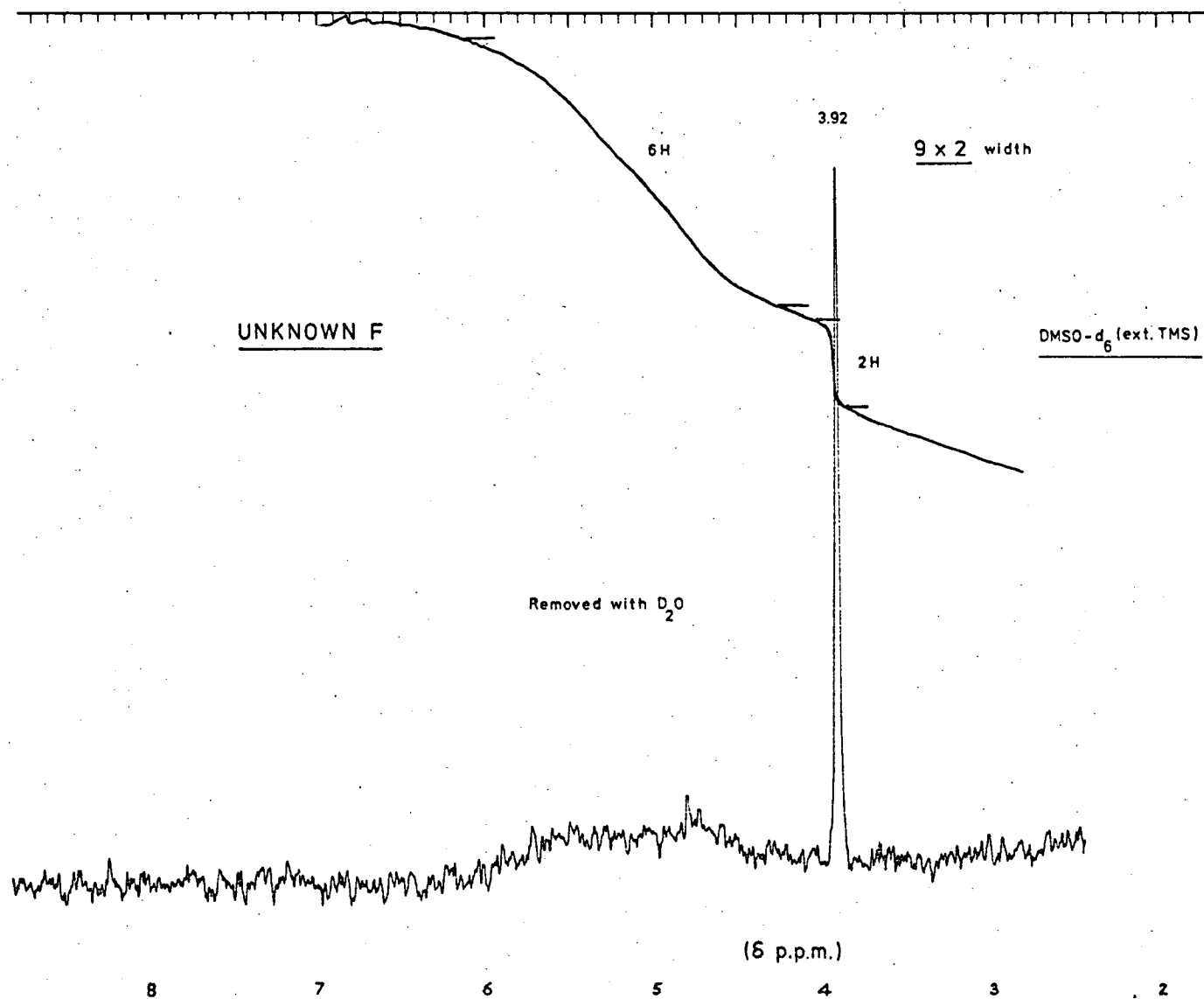


CHART 51. N.M.R. SPECTRUM of UNKNOWN F.



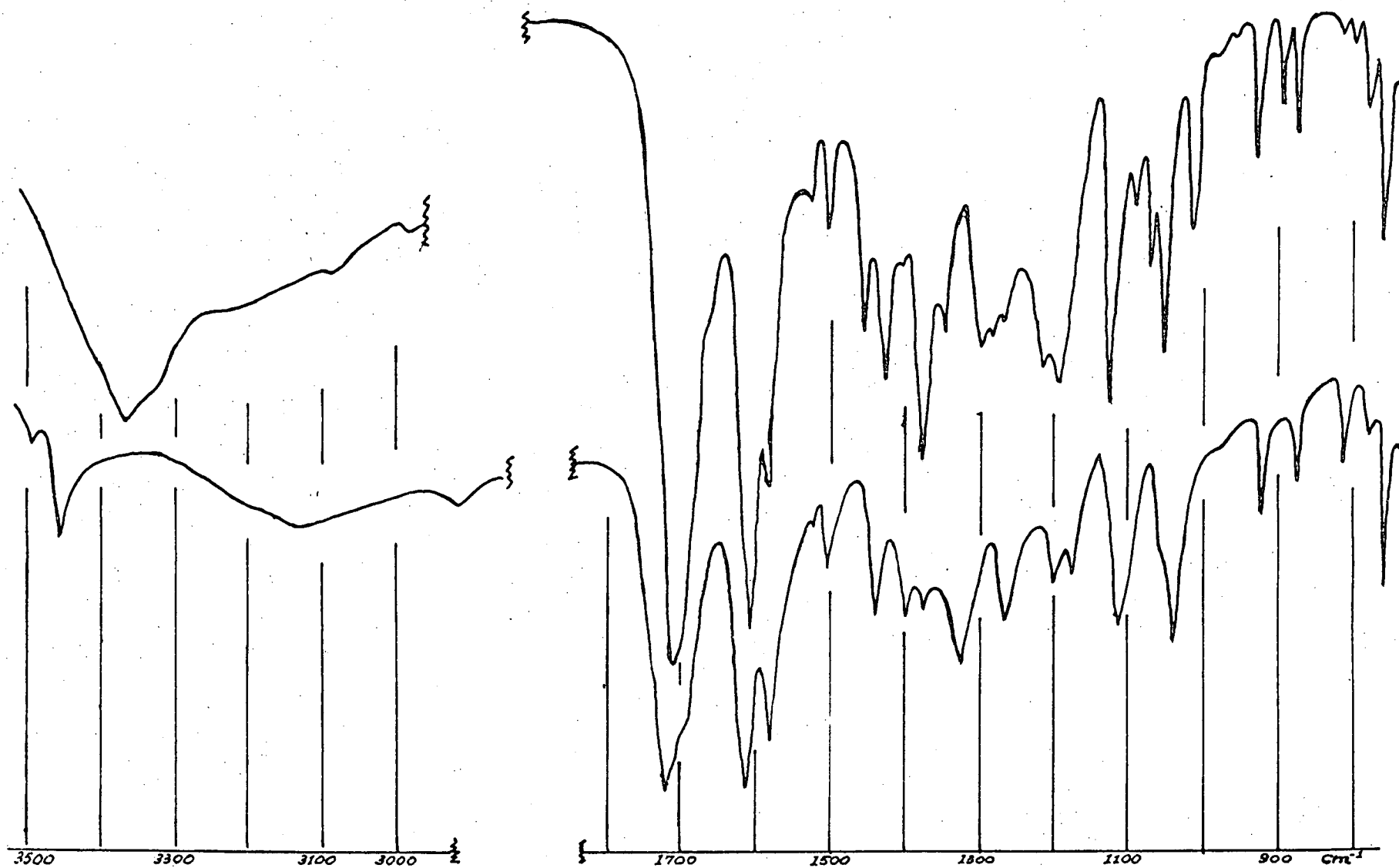


CHART 52. I.R. SPECTRA: ABOVE, Hillis' UNKNOWN F : BELOW, ELLAGIC ACID.

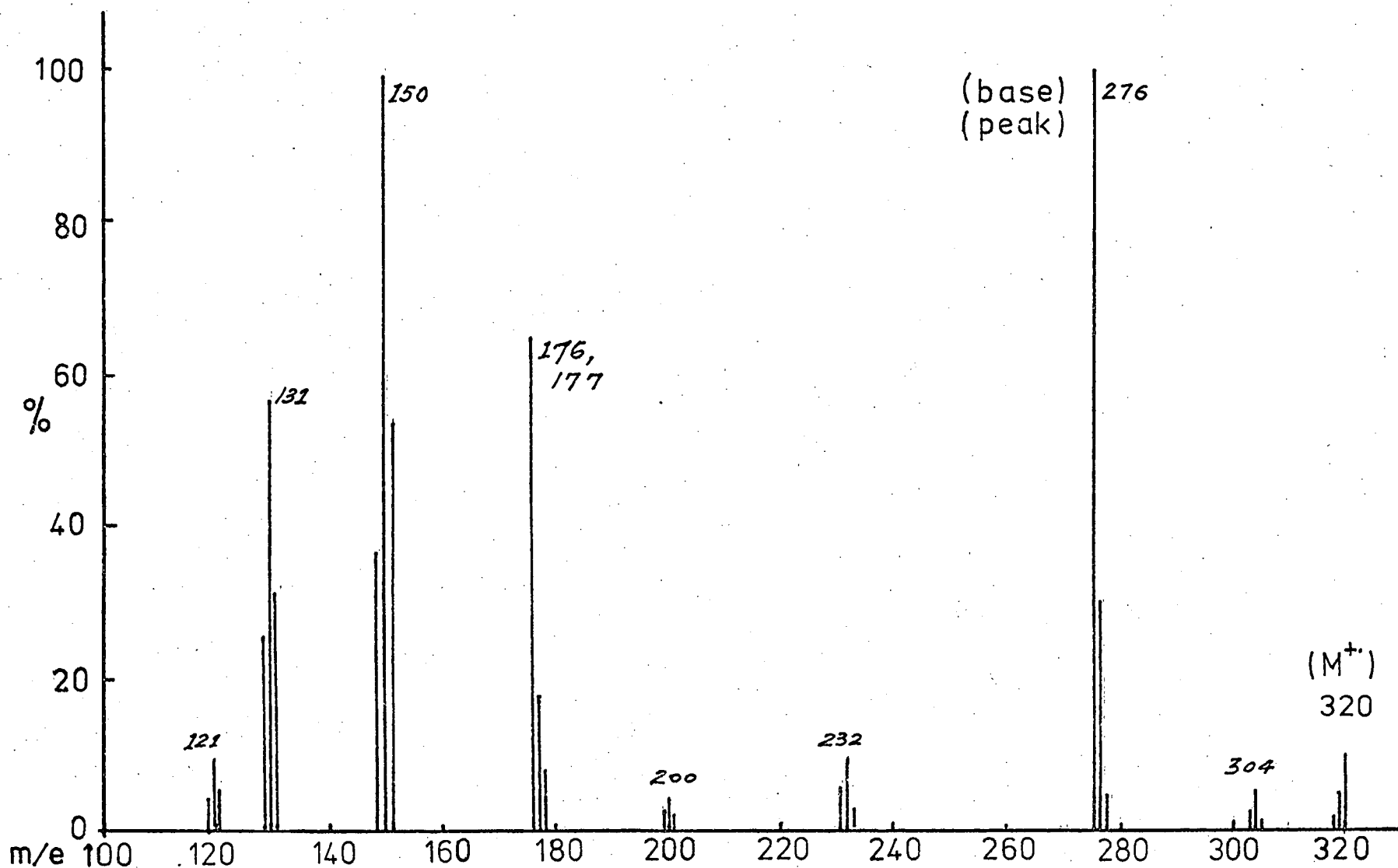


CHART 53. MASS SPECTRUM of UNKNOWN F.

### 3.2. DERIVATIVES.

Derivatives of various polyphenols have been made during the course of this study using one or other of the following procedures.

#### 3.2.1. TRIMETHYLSILYLATION.<sup>75c</sup>

A suitable quantity of the polyphenol (20 mg) was dissolved in pyridine (1 ml) (dried by storing over KOH pellets) in a 50 ml round-bottomed quickfit flask and hexamethyldisilazane (HMDS) (0.25 ml) and trimethylchlorosilane (TMCS) (0.25 ml) were added and the flask stopped and allowed to stand for 10-20 min. at room temperature. In the case of 5-hydroxy flavonoids the silylating solution was allowed to stand overnight before further work-up. Solvent and excess reagents having been evaporated under vacuum and the dry residue extracted with Analar chloroform with subsequent filtration of salts, the clear  $\text{CCl}_4$  solution was taken to dryness, re-dissolved in a quantity of solvent suitable for n.m.r. analysis with addition of a drop of each of the silylating reagents to ensure anhydrous conditions.

The original compound was regenerated unchanged in most instances by allowing the trimethylsilyl ether to stand overnight in 20% aqueous methanol with a drop of glacial acetic acid.

### 3.2.2. METHYLATION.

Diazomethane was generated in a fume hood either from nitrosomethylurea prepared according to the method of Arndt<sup>2</sup> or by distillation from an ether solution of p-tolylsulphonylmethylnitrosamide.<sup>18</sup> The diazomethane ether solution was added several times to a methanolic solution, or in some cases suspension, of the polyphenol over a period of several days, the solution being cooled to minimise the loss of diazomethane by evaporation.

Methylation of pinocembrin was carried out with dimethyl sulphate according to the method of Thomas.<sup>104</sup>

### 3.2.3. TRIDEUTERIOMETHYLATION.

Trideuteriomethylation of phenolic hydroxyls in certain polyphenols was attempted using the method devised by Bick et al.<sup>8</sup> for alkaloid hydroxyls. A diazomethane solution in light petrol dried over KOH pellets was extracted once with an equal volume of dimethylsulphoxide dried over molecular sieve, which removed some 75% of the diazomethane and the sulphoxide-diazomethane solution added to the polyphenol in dry dimethylsulphoxide containing a few drops of heavy water. The addition was repeated daily for several days.

This method of trideuteriomethylation achieved only a limited success with phenolic hydroxyls. The 5-hydroxyl of pinocembrin and the hydroxyls of unknown A were only partially trideuteriomethylated and the yields in both cases were poor.

#### 3.2.4. ACETYLATION.

To a small quantity of compound (20 mg) in a round-bottomed quickfit flask fitted for refluxing was added sodium bicarbonate (5 mg), acetic anhydride (re-distilled) (0.5 ml) and glacial acetic acid (0.2 ml) and the solution was refluxed for 15-30 minutes after which it was poured into twice the volume of water with subsequent stirring and warming to decompose excess anhydride. The solution was then extracted with chloroform until the extracts were colourless and these extracts bulked and concentrated to the point where cloudiness appeared. Upon standing crystals of the acetate settled out and were crystallized from chloroform.

An alternative method of acetylation used during this work has been that of Occolowitz<sup>84</sup> in which a small quantity of polyphenol (20 mg) was refluxed with acetic anhydride (0.2 g) and anhydrous pyridine (1 ml) for 5 min. The mixture was cooled and poured into ice water (1.5 ml). After washing, the crude ester was crystallized from ethanol.

#### 3.2.5. PEROXIDE OXIDATION OF FLAVONOL-3-GLYCOSIDES.<sup>22</sup>

The flavonol glycoside (1 mg) was suspended in water (0.2 ml) containing 0.1M ammonia (0.01 ml). Hydrogen peroxide (30%; 40 ml) was added and after four hours at room temperature the solution was treated with a little palladium as catalyst to decompose excess peroxide. After 20 hours ammonia (0.880; 50 ml) was

added and the solution warmed for five minutes in a boiling water bath. The resulting solution was first used directly for paper chromatographic analysis and then extracted with ether to separate the aglycone and the concentrated ether solution and remaining aqueous solution separately co-chromatographed with authentic samples of appropriate compounds.

### 3.2.6. ACID HYDROLYSIS OF GLYCOSIDES.<sup>83</sup>

The glycoside (1 mg) was heated at 100°C with 0.5M hydrochloric acid (0.5 ml) for 30 minutes and the resultant solution examined by paper chromatography, with solutions of authentic samples.

### 3.3. ROTATIONS, ANALYSES AND MELTING POINTS.

Optical rotations of the flavanones, the flavan-3-ol and the rotation of rutin (quercetin-3-rhamnosylglucoside) were found using a Bellingham-Stanley polarimeter on solutions of 2% or 5% concentration depending on the quantity of material available, in a cell of 10 cm length and volume 2 ml.

Microanalyses are by the Australian Microanalytical Service, Melbourne, and the Australian National University.

Melting points are uncorrected.

Samples were dried at 0.05 mm Hg over phosphorus pentoxide at temperatures specified in the text. All evaporations were carried out under reduced pressure.

### 3.4. EXPERIMENTAL DATA.

In purely qualitative tests in dilute solution the location of maxima in u.v. spectra is sufficient so extinction values are not reported for every compound.

The data are given first for polyphenols of known structure; second, for polyphenols called by Hillis unknowns.

#### 3.4.1. POLYPHENOLS OF KNOWN STRUCTURE.

##### 3.4.1.1. Hydroxybenzoic acids.

##### 3.4.1.1.1. Gallic acid (3,4,5-trihydroxybenzoic acid).

Gallic acid had  $R_f$  0.58 BAW (612), 0.65 in F, 0.42 in 6HOAc. It crystallized from water in long white needles, m.p.  $240^\circ$ - $242^\circ$  (dec.) (lit.<sup>69c</sup>  $225^\circ$ - $250^\circ$  dec.), undepressed on admixture with an authentic sample;  $\lambda_{\max}$  210, 272, in MeOH/NaOMe:370, in MeOH/ $AlCl_3$ :308, in MeOH/ $AlCl_3$  + HCl:273, in MeOH/NaOAc:261, in MeOH/NaOAc +  $H_3BO_3$ :286 nm;  $\nu_{\max}$  3457, 3349, 3256, 1698 (carboxyl C=O), 1610, 1536, 1247, 1025, 865, 765  $cm^{-1}$ ;  $\delta$  (DMSO- $d_6$ ) 7.29 (s, 2H, ArH), 10.0 (broad multiplet, 4H, OH,  $CO_2H$ , removed with  $D_2O$ ); the mass spectrum is shown in Chart 11, page 55. (Found: C, 49.0; H, 3.4 Calc. for  $C_7H_6O_5$ : C, 49.4; H, 3.6%).

##### 3.4.1.1.2. Gentisic acid (2,5-dihydroxybenzoic acid).

Gentisic acid had  $R_f$  0.82 in BAW (612), 0.86 in BAW (415), 0.86 in F, 0.62 in 6HOAc, 0.66 in 15HOAc. It crystallized as needles from water, m.p.  $197^\circ$ - $200^\circ$  (lit.<sup>69b</sup>  $199^\circ$ - $200^\circ$ );  $\lambda_{\max}$  in MeOH 237 (sh) 335, in MeOH/NaOMe:342, in MeOH/NaOAc +  $H_3BO_3$ :335 nm;  $\nu_{\max}$  3290, 3130, 1660 (carboxyl C=O), 1610, 1585, 1270, 1230, 1175, 930,

840, 785  $\text{cm}^{-1}$ ;  $\delta$  (DMSO- $\text{d}_6$ ) 7.08 (d, 1H,  $\underline{\text{H}}_3$ ,  $J_{\text{meta}} = 3$  Hz), 7.28 (q, 1H,  $\underline{\text{H}}_4$ ,  $J_{\text{meta}} = 3$  Hz,  $J_{\text{ortho}} = 9.5$  Hz), 7.55 (d, 1H,  $\underline{\text{H}}_6$ ,  $J_{\text{meta}} = 3$  Hz), 11.04 (m, 3H,  $\underline{\text{OH}}$ ,  $\text{CO}_2\underline{\text{H}}$ , removed with  $\text{D}_2\text{O}$ ); the mass spectrum is shown in Chart 11, page 55. (Found: C, 54.4; H, 4.0. Calc. for  $\text{C}_7\text{H}_6\text{O}_4$ : C, 54.5; H, 3.9%).

### 3.4.1.1.3. Protocatechuic acid (3,4-dihydroxybenzoic acid).

Protocatechuic acid had  $R_f$  0.68 in BAW (612), 0.70 in BAW (415), 0.70 in F, 0.60 in 6HOAc and 0.66 in 15HOAc. It crystallized from water in off-white needles, m.p.  $194^\circ$ - $196^\circ$  (dec.) (lit.<sup>69a</sup>  $195^\circ$ - $196^\circ$  dec.);  $\lambda_{\text{max}}$  in MeOH: 259, 294, in MeOH/NaOMe: 276, 299, in MeOH/ $\text{AlCl}_3$ : 274, 388 (sh), in MeOH/ $\text{AlCl}_3 + \text{HCl}$ : 261, 295, in MeOH/NaOAc: 251, 289, in MeOH/NaOAc+ $\text{H}_3\text{BO}_3$ : 267, 302 nm;  $\nu_{\text{max}}$  3327, 3182, 1672 (carboxyl C=O), 1605, 1590, 1522, 1290, 1127, 1090, 921, 820, 764  $\text{cm}^{-1}$ ;  $\delta$  (DMSO- $\text{d}_6$ ) 7.16 (d, 1H,  $\underline{\text{H}}_6$ ,  $J_{\text{ortho}} = 9$  Hz), 7.69 (d, 1H,  $\underline{\text{H}}_5$ ,  $J_{\text{ortho}} = 9$  Hz), 7.73 (s, 1H,  $\underline{\text{H}}_2$ ), 10.34 (multiplet, 3H,  $\underline{\text{OH}}$ ,  $\text{CO}_2\underline{\text{H}}$ , removed with  $\text{D}_2\text{O}$ ); the mass spectrum is shown in Chart 11, page 55. (Found: C, 54.3; H, 4.1. Calc. for  $\text{C}_7\text{H}_6\text{O}_4$ : C, 54.5; H, 3.9%).

### 3.4.1.2. FLAVONOLS.

#### 3.4.1.2.1. Quercetin (3',4',5,7-tetrahydroxyflavon-3-ol).

Quercetin had  $R_f$  0.64 in BAW (612), 0.40 in F, 0.56 in TBA and 0.03 in 15HOAc. It crystallized from



dilute alcohol as yellow needles, m.p.  $314^{\circ}$ - $316^{\circ}$  (lit.<sup>35h</sup>  $316^{\circ}$ - $318^{\circ}$ ); the penta-acetate recrystallized from methanol, m.p.  $198^{\circ}$ - $199^{\circ}$  (lit.<sup>35h</sup>  $200^{\circ}$ ); quercetin had  $\lambda_{\max}$  255, 259 (sh) 300 (sh) 370, in MeOH/NaOMe: 250 (sh), 320 (dec.), in MeOH/ $\text{AlCl}_3$ : 272, 304 (sh), 332, 458, in MeOH/ $\text{AlCl}_3$ +HCl: 265, 300 (sh), 360, 428, in MeOH/NaOAc: 255 (sh), 274, 329, 390 (dec.), in MeOH/NaOAc+ $\text{H}_3\text{BO}_3$ : 260, 303 (sh), 388 nm:  $\nu_{\max}$  3400, 3310, 1666 (chelated C=O), 1610, 1560, 1270, 1170, 864, 794  $\text{cm}^{-1}$ ;  $\delta(\text{DMSO}-d_6)$ , 6.22 (d, 1H,  $\underline{\text{H}}_6$ ,  $J_{\text{meta}} = 3$  Hz), 6.42 (d, 1H,  $\underline{\text{H}}_8$ ,  $J_{\text{meta}} = 3$  Hz), 6.90 (d, 1H,  $\underline{\text{H}}_5$ ,  $J_{\text{ortho}} = 9$  Hz), 7.62 (t, 1H,  $\underline{\text{H}}_6$ ,  $J_{\text{meta}} = 3$  Hz,  $J_{\text{ortho}} = 9$  Hz), 7.80 (m, 1H,  $\underline{\text{H}}_2$ ), 9.06 (broad m, 2H,  $\text{OH}_{3,4}$ , removed with  $\text{D}_2\text{O}$ ), 9.42 (m, 1H,  $\text{OH}_3$ , removed with  $\text{D}_2\text{O}$ ), 10.86 (m, 1H,  $\text{OH}_7$ , removed with  $\text{D}_2\text{O}$ ), 12.60 (s, 1H,  $\text{OH}_5$ , partially removed with  $\text{D}_2\text{O}$ ); for the penta-acetate  $\delta(\text{CDCl}_3)$ , 6.15 (d, 1H,  $\underline{\text{H}}_6$ ,  $J_{\text{meta}} = 3$  Hz), 6.45 (d, 1H,  $\underline{\text{H}}_8$ ,  $J_{\text{meta}} = 3$  Hz), 6.75 (d, 1H,  $\underline{\text{H}}_5$ ,  $J_{\text{ortho}} = 9$  Hz), 7.55 (q, 1H,  $\underline{\text{H}}_6$ ,  $J_{\text{meta}} = 3$  Hz,  $J_{\text{ortho}} = 9$  Hz), 7.72 (d, 1H,  $\underline{\text{H}}_2$ ,  $J_{\text{meta}} = 3$  Hz); mass spectrum m/e (rel. intensity) 302 (24), 152 (15), 153 (32), 137 (46). (Found: C, 59.4; H, 3.3. Calc. for  $\text{C}_{15}\text{H}_{10}\text{O}_7$ : c, 59.6; H, 3.3%).

#### 3.4.1.2.2. Myricetin (3',4',5,5',7-pentahydroxyflavon-3-ol).

Myricetin had  $R_f$  0.41 in BAW (612), 0.43 in BAW (415), 0.31 in TBA, 0.00 in 6HOAc and 0.01 in 15HOAc. It crystallized as yellow-green needles from aqueous

alcohol, m.p.  $354^{\circ}$ - $357^{\circ}$  (lit.<sup>35i</sup>  $357^{\circ}$ - $360^{\circ}$ ), undepressed on admixture with an authentic sample;  $\lambda_{\max}$  in MeOH: 254, 270 (sh), 300 (sh), 372, in MeOH/NaOMe: 262 (sh), 284 (sh), 322, 420 (dec.), in MeOH/ $\text{AlCl}_3$ : 270, 315 (sh), 450, in MeOH/ $\text{AlCl}_3$ +HCl: 266, 275 (sh), 307 (sh), 358 (sh), in MeOH/NaOAc: 270, 335 (dec.), in MeOH/NaOAc+ $\text{H}_3\text{BO}_3$ : 256, 304 (sh), 392 nm;  $\nu_{\max}$  3445, 3315, 1655 (chelated C=O), 1605, 1550, 1270, 1170, 858 (isolated ArH)  $\text{cm}^{-1}$ ;  $\delta$  (DMSO- $\text{d}_6$ ), 6.25 (d, 1H,  $\underline{\text{H}}_6$ ,  $J_{\text{meta}} = 2.5$  Hz), 6.48 (d, 1H,  $\underline{\text{H}}_8$ ,  $J_{\text{meta}} = 2.5$  Hz), 7.35 (s, 2H,  $\underline{\text{H}}_{2,6}$ ), 9.35 (m, 1H,  $\underline{\text{OH}}_4$ , removed with  $\text{D}_2\text{O}$ ), 9.88 (m, 1H,  $\underline{\text{OH}}_3$ , removed with  $\text{D}_2\text{O}$ ), 10.87 (m, 1H,  $\underline{\text{OH}}_7$ , removed with  $\text{D}_2\text{O}$ ), 12.55 (s, 1H,  $\underline{\text{OH}}_5$  chelated, partially removed with  $\text{D}_2\text{O}$ ); mass spectrum m/e (rel. intensity) 318 (72), 302 (24), 153 (67), 150 (16), 137 (45), 125 (28), 109 (100) corresponding to the fragmentation shown in Chart 15, page 59. (Found: C, 56.4; H, 3.1. Calc. for  $\text{C}_{15}\text{H}_{10}\text{O}_8$ : C, 56.6; H, 3.2%).

#### 3.4.1.2.3. Kaempferol (4',5,7-trihydroxyflavon-3-ol).

Kaempferol had  $R_f$  0.82 in BAW (612), 0.80 in BAW (415), 0.55 in F, 0.00 in 6HOAc and 0.04 in 15HOAc. It crystallized as pale yellow needles from aqueous alcohol, m.p.  $274^{\circ}$ - $275^{\circ}$  (lit.<sup>35g</sup>  $277^{\circ}$ - $279^{\circ}$ ), undepressed on admixture with an authentic sample;  $\lambda_{\max}$  in MeOH: 253 (sh), 295 (sh), 322 (sh), 366, in MeOH/NaOMe: 277, 316, 416, in MeOH/ $\text{AlCl}_3$ : 261 (sh), 267, 302 (sh), 350, 422, in MeOH/ $\text{AlCl}_3$ +HCl: 256 (sh), 268, 303 (sh), 349, 422, in MeOH/NaOAc: 275, 303, 388, in MeOH/NaOAc+ $\text{H}_3\text{BO}_3$ : 265, 297

(sh), 320 (sh), 370 nm;  $\nu_{\max}$  3420, 3300, 1664 (chelated C=O), 1605, 1558, 1510, 1270, 1170, 864 (strong)  $\text{cm}^{-1}$ ;  $\delta$  (DMSO- $\text{d}_6$ , int. TMS), 6.26 (d, 1H,  $\underline{\text{H}}_6$ ,  $J_{\text{meta}} = 2.5$  Hz), 6.48 (d, 1H,  $\underline{\text{H}}_8$ ,  $J_{\text{meta}} = 2.5$  Hz), 7.00 (d, 2H,  $\underline{\text{H}}_{3',5'}$ ,  $J_{\text{ortho}} = 8.5$  Hz), 8.12 (d, 2H,  $\underline{\text{H}}_{2',6'}$ ,  $J_{\text{ortho}} = 8.5$  Hz), 9.45 (m, 1H,  $\underline{\text{OH}}_3$ , removed with  $\text{D}_2\text{O}$ ), 10.18 (m, 1H,  $\underline{\text{OH}}_{4'}$ , removed with  $\text{D}_2\text{O}$ ), 10.90 (m, 1H,  $\underline{\text{OH}}_7$ , removed with  $\text{D}_2\text{O}$ ), 12.60 (s, 1H,  $\underline{\text{OH}}_5$ , partially removed with  $\text{D}_2\text{O}$ ); the trimethylsilyl ether gave  $\delta$  ( $\text{CDCl}_3$ ), 6.15 (d, 1H,  $\underline{\text{H}}_6$ ,  $J_{\text{meta}} = 2.5$  Hz), 6.45 (d, 1H,  $\underline{\text{H}}_8$ ,  $J_{\text{meta}} = 2.5$  Hz), 6.84 (d, 2H,  $\underline{\text{H}}_{3',5'}$ ,  $J_{\text{ortho}} = 8.5$  Hz), 7.95 (d, 2H,  $\underline{\text{H}}_{2',6'}$ ,  $J_{\text{ortho}} = 8.5$  Hz), 12.40 (s, 1H,  $\underline{\text{OH}}_5$ , not trimethylsilylated); mass spectrum  $m/e$  (rel. intensity) 286 (24), 152 (32), 121 (44), 118 (35), 105 (36), 93 (29). (Found: C, 62.7; H, 3.6. Calc. for  $\text{C}_{15}\text{H}_{10}\text{O}_6$ : C, 62.9; H, 3.5%).

### 3.4.1.3. FLAVONOL GLYCOSIDES.

#### 3.4.1.3.1. Rutin (quercetin-3-rhamnosylglucoside).

Rutin had  $R_f$  0.41 in BAW (415), 0.46 in TBA, 0.40 in EPW, 0.42 in 15HOAc and 0.52 in PhOH. It crystallised as pale yellow needles from dilute alcohol, m.p.  $190^\circ\text{--}192^\circ$  (dried at  $100^\circ$  in vacuo) (lit.<sup>69e</sup>  $188^\circ\text{--}190^\circ$ ), undepressed on admixture with an authentic sample, and had  $[\alpha]_{\text{D}}^{20} = -30^\circ$  (c, 1.0 in MeOH) (lit.<sup>35f</sup>  $-30^\circ$ );  $\lambda_{\max}$  in MeOH: 255, 265 (sh), 295 (sh), 356, in MeOH/NaOMe: 272, 326, 409 (no dec.), in MeOH/ $\text{AlCl}_3$ : 273, 300 (sh), 432, in MeOH/ $\text{AlCl}_3 + \text{HCl}$ : 271, 300, 362 (sh), 402, in

MeOH/NaOAc: 270, 324, 390, in MeOH/NaOAc+H<sub>3</sub>BO<sub>3</sub>: 260, 298, 386 nm;  $\nu_{\max}$  3374 (broad), 1658 (chelated C=O), 1605, 1550, 1204; 1200, 1030, 900, 600 cm<sup>-1</sup>;  $\delta$  (DMSO-d<sub>6</sub>), 1.0 (d, 3H, CH<sub>3</sub>rhamnose), 3.25 (m, 10H, sugar protons), 4.45 (s, 1H, H rhamnosyl), 5.40 (m, 1H, H glucosyl), 6.25 (d, 1H, H<sub>6</sub>, J<sub>meta</sub> = 3 Hz), 6.45 (d, 1H, H<sub>8</sub>, J<sub>meta</sub> = 3 Hz), 6.92 (d, 1H, H<sub>5</sub>, J<sub>ortho</sub> = 9 Hz), 7.68 (m, 2H, H<sub>2</sub>,<sub>6</sub>), 9.05 (m, 2H, OH<sub>3</sub>,<sub>4</sub>, removed with D<sub>2</sub>O), 10.78 (m, 1H, OH<sub>7</sub>, removed with D<sub>2</sub>O), 12.60 (s, 1H, OH<sub>5</sub>, chelated, partially removed with D<sub>2</sub>O); the trimethylsilyl ether had  $\delta$  (CDCl<sub>3</sub>), 0.90 (d, 3H, CH<sub>3</sub>rhamnose), 3.30 (m, 10H, sugar protons), 4.15 (s, 1H, H rhamnosyl), 5.75 (m, 1H, H glucosyl), 6.10 (d, 1H, H<sub>6</sub>, J<sub>meta</sub> = 3 Hz), 6.35 (d, 1H, H<sub>8</sub>, J<sub>meta</sub> = 3 Hz), 6.80 (d, 1H, H<sub>5</sub>, J<sub>ortho</sub> = 9 Hz), 7.38 (m, 2H, H<sub>2</sub>,<sub>6</sub>). (Found: C, 48.5; H, 5.3; Calc. for C<sub>27</sub>H<sub>30</sub>O<sub>16</sub>·3H<sub>2</sub>O: C, 48.8; H, 5.5%).

#### 3.4.1.3.2. Quercitrin (quercetin-3-rhamnoside).

Quercitrin had R<sub>f</sub> 0.70 in BAW (415), 0.68 in TBA, 0.48 in 15HOAc and 0.56 in PhOH. It crystallized from water as yellow needles, m.p. 180°-181° (lit.<sup>69d</sup> 182°-184°), undepressed on admixture of an authentic sample;  $\lambda_{\max}$  in MeOH: 255, 265 (sh), 293 (sh), 348, in MeOH/NaOMe: 270, 324, 393 (no dec.), in MeOH/AlCl<sub>3</sub>: 274, 304 (sh), 333, 432, in MeOH/AlCl<sub>3</sub>+HCl: 272, 303 (sh), 350, 400, in MeOH/NaOAc: 272, 320 (sh), 372, in MeOH/NaOAc+H<sub>3</sub>BO<sub>3</sub>: 260, 300 (sh), 365 nm;  $\nu_{\max}$  3385 (broad),

1645, (chelated C=O), 1602, 1550, 1200, 900, 600  $\text{cm}^{-1}$ ;  $\delta$  (DMSO- $\text{d}_6$ ), 1.1 (d, 3H,  $\text{CH}_3$  rhamnosyl), 3.40 (m, 3H,  $\text{H}_{3,4,5}$  rhamnosyl), 4.60 (m, 1H,  $\text{H}_2$  rhamnosyl), 5.35 (s, 1H,  $\text{H}_1$  rhamnosyl), 6.22 (d, 1H,  $\text{H}_6$ ,  $J_{\text{meta}} = 3$  Hz), 6.38 (d, 1H,  $\text{H}_8$ ,  $J_{\text{meta}} = 3$  Hz), 6.89 (d, 1H,  $\text{H}_5$ ,  $J_{\text{ortho}} = 9$  Hz), 7.30 (m, 1H,  $\text{OH}_7$ , removed with  $\text{D}_2\text{O}$ ), 12.58 (s, 1H,  $\text{OH}_5$ , partially removed with  $\text{D}_2\text{O}$ ); the trimethylsilyl ether had  $\delta$  ( $\text{CDCl}_3$ ), 0.85 (d, 3H,  $\text{CH}_3$  rhamnosyl), 3.30 (m, 3H,  $\text{H}_{3,4,5}$  rhamnosyl), 4.20 (m, 1H,  $\text{H}_2$  rhamnosyl), 5.05 (s, 1H,  $\text{H}_1$  rhamnosyl), 6.05 (d, 1H,  $\text{H}_5$ ,  $J_{\text{ortho}} = 9$  Hz), 7.25 (m, 2H,  $\text{H}_{2,6}$ ). (Found: C, 51.9; H, 4.9. Calc. for  $\text{C}_{21}\text{H}_{20}\text{O}_{11} \cdot 2\text{H}_2\text{O}$ : C, 52.1; H, 4.9%).

#### 3.4.1.3.3. Isoquercitrin (quercetin-3-glucoside).

Isoquercitrin had  $R_f$  0.58 in BAW (612), 0.50 in TBA, 0.20 in 6HOAc, 0.40 in 15HOAc and 0.50 in PhOH. It crystallized as pale yellow needles from water, m.p.  $222^\circ$ - $224^\circ$  (lit.<sup>46</sup>  $228^\circ$ - $229^\circ$ );  $\lambda_{\text{max}}$  in MeOH: 258, 268 (sh), 357, in MeOH/NaOMe: 272, 326, 410 (no dec.), in MeOH/ $\text{AlCl}_3$ : 273, 301 (sh), 330, 426, in MeOH/ $\text{AlCl}_3$ +HCl: 269, 301 (sh), 349, 403, in MeOH/NaOAc: 257 (sh), 273, 315, 368, in MeOH/NaOAc+ $\text{H}_3\text{BO}_3$ : 261, 297 (sh), 370 nm; oxidation products quercetin and glucose had identical  $R_f$  when co-chromatographed with authentic samples. (Found: C, 51.1; H, 4.7. Calc. for  $\text{C}_{21}\text{H}_{20}\text{O}_{12} \cdot 1\frac{1}{2}\text{H}_2\text{O}$ : C, 51.3; H, 4.7%).

#### 3.4.1.3.4. Hyperin (quercetin-3-galactoside).

Hyperin had  $R_f$  0.55 in BAW (415), 0.42 in TBA, 0.33 in 15HOAc and 0.56 in PhOH. It recrystallized from aqueous alcohol as pale yellow needles, m.p.  $235^\circ$ - $237^\circ$  (dec.) (lit.<sup>35d</sup>  $237^\circ$ - $238^\circ$ );  $\lambda_{\max}$  in MeOH: 259, 267, 293 (sh), 356, in MeOH/NaOMe: 272, 328, 400 (no dec.), in MeOH/ $AlCl_3$ : 274, 304 (sh), 425, in MeOH/ $AlCl_3$ +HCl: 271, 304, 355 (sh), 398, in MeOH/NaOAc: 268, 320 (sh), 370 (sh), in MeOH/NaOAc+ $H_3BO_3$ : 279, 310 (sh), 376 nm; peroxide oxidation yielded quercetin with melting point undepressed on admixture with an authentic sample and galactose with  $R_f$  in two solvents identical with an authentic sugar and located by spraying with p-anisidine hydrochloride followed by heating.

#### 3.4.1.3.5. Cannabiscitrin (myricetin-3-glucoside).

Cannabiscitrin had  $R_f$  0.47 in BAW (415), 0.43 in TBA, 0.24 in 15HOAc and 0.32 in PhOH. It crystallized from aqueous alcohol as yellow needles, m.p.  $218^\circ$ - $220^\circ$ ;  $\lambda_{\max}$  in MeOH: 267, 290 (sh), 367 (sh), in MeOH/NaOMe: 272 (sh), 321, 396 (no dec.), in MeOH/ $AlCl_3$ : 271, 307 (sh), 408, in MeOH/ $AlCl_3$ +HCl: 271, 305 (sh), 355, 400, in MeOH/NaOAc: 270, 300 (sh), 340, 425, in MeOH/NaOAc+ $H_3BO_3$ : 262, 296 (sh), 395 nm; peroxide oxidation yielded myricetin with melting point undepressed on admixture with an authentic sample and glucose identified by  $R_f$  values with an authentic sugar.

### 3.4.1.3.6. Afzelin (kaempferol-3-rhamnoside).

Afzelin had  $R_f$  0.80 in BAW (612), 0.82 in BAW (415), 0.33 in 6HOAc and 0.68 in PhOH. It crystallized from alcohol as yellow needles, m.p.  $170^{\circ}$ - $172^{\circ}$  (lit.<sup>71</sup>  $172^{\circ}$ - $174^{\circ}$ );  $\lambda_{\max}$  in MeOH: 267, 315 (sh), 348, in MeOH/NaOMe: 272, 300 (sh), 350 (sh), 399 (no dec.), in MeOH/ $AlCl_3$ : 255 (sh), 274, 300, 346, in MeOH/ $AlCl_3$ +HCl: 274, 298 (sh), 348, 398, in MeOH/NaOAc: 273, 316 (sh), in MeOH/NaOAc+ $H_3BO_3$ : 269, 316 (sh), 352 nm; peroxide oxidation of afzelin yielded kaempferol and rhamnose identified by co-chromatography with authentic samples.

### 3.4.1.4. Ellagic acid.

Ellagic acid had  $R_f$  0.32 in BAW (415), 0.34 in BAW (612), 0.02 in 6HOAc and 0.05 in 15HOAc. It crystallized as a fine yellow powder from aqueous alcohol, m.p.  $> 360^{\circ}$  (lit.<sup>53b</sup>  $> 360^{\circ}$ ), undepressed on admixture of an authentic sample;  $\lambda_{\max}$  in MeOH: 244 (sh), 255, 265 (sh), 366, in MeOH/NaOMe: 245, 284, 350 (sh) (no dec.), in MeOH/ $AlCl_3$ : 244 (sh), 269, 385, in MeOH/ $AlCl_3$ +HCl: 254, 360, in MeOH/NaOAc: 244 (sh), 254, 277, 365, in MeOH/NaOAc+ $H_3BO_3$ : 244, 266, 318 (sh), 378 nm;  $\nu_{\max}$  3460, 3140, (bound OH), 1720 (non-chelated C=O), 1612, 1580, 1510, 1270, 1200, 1114 (C-O stretch), 1043 (C-O stretch), 928, 880 (isolated ArH),  $818\text{ cm}^{-1}$ ;  $\delta$  ( $C_5D_5N$ ,  $80^{\circ}$ ), 8.01 (s, sH,  $H_{5,5}$ ), 5.08 (d, 2H,  $OH_{4,4}$ , removed with  $D_2O$ ,  $J = 25\text{ Hz}$ ), 4.25 (d, 2H,  $OH_{3,3}$ ,  $J = 25\text{ Hz}$ , removed with

D<sub>2</sub>O); for the trimethylsilyl ether,  $\delta$ (CDCl<sub>3</sub>), 7.63 (s, 2H,  $\underline{H}_{5,5'}$ ); mass spectrum m/e (rel. intensity) 302 (100), 286 (4), 273 (7), 246 (9), 228 (7), 190 (7), 162 (10), corresponding to the fragmentation shown in Chart 16, page 60. (Found: C, 55.4; H, 2.0. Calc. for C<sub>14</sub>H<sub>6</sub>O<sub>8</sub>: C, 55.6; H, 2.0%).

3.4.1.5. (+)-Catechin (3,5,7,3',4'-pentahydroxyflavan).

(+)-Catechin had R<sub>f</sub> 0.68 in BAW (612), 0.60 in F, 0.45 in 6HOAc and 0.60 in 15HOAc. It crystallized as fawn needles from dilute acetic acid, m.p. 173°-175° (lit.<sup>69h</sup> 175°-177°), undepressed on admixture with an authentic sample, and had  $[\alpha]_D^{18} = +16.5$  (c. 1.0 in 50% acetone, water);  $\lambda_{\max}$  in MeOH: 280, in MeOH/NaOMe: 292, 430 (dec.), in MeOH/AlCl<sub>3</sub>: 280, 286, in MeOH/AlCl<sub>3</sub>+HCl: 280, in MeOH/NaOAc: 283, 295 (sh), in MeOH/NaOAc+H<sub>3</sub>BO<sub>3</sub>: 287 nm;  $\nu_{\max}$  3488, 3386, 3210, 1610, 1520, 1290, 1152, 1032, 868, 828, 798 cm<sup>-1</sup>;  $\delta$ (DMSO-d<sub>6</sub>), 3.70 (m, 2H,  $\underline{H}_{4eq}$ ,  $\underline{H}_{4ax}$ ), 4.50 (m, 1H,  $\underline{H}_3$ ), 4.96 (d, 1H,  $\underline{H}_2$ , J<sub>2,3trans</sub> = 8 Hz), 5.72 (d, 1H,  $\underline{H}_6$ , J<sub>meta</sub> = 3 Hz), 6.02 (d, 1H,  $\underline{H}_8$ , J<sub>meta</sub> = 3 Hz),  $\delta$ (C<sub>5</sub>D<sub>5</sub>N), for ABMX signals, 3.31 (q, 1H,  $\underline{H}_{4ax}$ , J<sub>3,4ax</sub> = 9 Hz, J<sub>4eq,4ax</sub> = 16.3 Hz), 3.70 (q, 1H,  $\underline{H}_{4eq}$ , J<sub>3,4eq</sub> = 5 Hz, J<sub>4eq,4ax</sub> = 16.3 Hz), 4.59 (octet, 1H,  $\underline{H}_3$ , J<sub>3,4eq</sub> + J<sub>3,4ax</sub> = 14 Hz, J<sub>2,3trans</sub> = 8 Hz), 5.20 (d, 1H,  $\underline{H}_2$ , J<sub>2,3trans</sub> = 8 Hz); mass spectrum m/e (rel. intensity) 290 (27), 152 (55), 149 (16), 139 (100), 138 (10), 123 (92), according to the fragmentation pattern in Chart 21, page 65. (Found: C, 61.8;



H, 4.9. Calc. for  $C_{15}H_{14}O_6$ : C, 62.1; H, 4.9%).

### 3.4.2. POLYPHENOLS OF UNKNOWN STRUCTURE (HILLIS' UNKNOWNNS).<sup>48</sup>

#### 3.4.2.1. Unknown D - Flavanones.

##### 3.4.2.1.1. Pinocembrin (E. delegatensis and E. sieberi).

Pinocembrin had  $R_f$  0.91 in BAW, 0.05 in HOAc, 0.86 in CEF, and 0.68 in MCP. It crystallized from methanol in short white needles, m.p.  $192^\circ$ - $193^\circ$  (lit.<sup>69f</sup>  $194^\circ$ - $195^\circ$ ), undepressed on admixture with synthetic pinocembrin, and had  $[\alpha]_D^{15} -45.3^\circ$  (c, 0.9 in acetone) (lit.<sup>25</sup>  $-46^\circ$ );  $\lambda_{max}$  289, 324 (sh), nm,  $\log \epsilon_{max}$  4.33, 4.02;  $\lambda_{max}$  in MeOH/ $AlCl_3$ : 310, in MeOH/NaOAc: 323 nm;  $\delta$  3.07 ( $H_{3eq}$ , q), 3.52 ( $H_{3ax}$ , 1), 5.90 ( $H_2$ , 1), 6.28 ( $H_6+H_6$ , s), 7.80 (ring-B H, m); 11.20, 12.48 (7-OH, m, 5-OH, s, removed with  $D_2O$ );  $J_{2,3eq}$  3.0,  $J_{2,3ax}$  13.0,  $J_{3eq,3ax}$  17.0. (Found: C, 70.1; H, 4.7. Calc. for  $C_{15}H_{12}O_4$ : C, 70.3; H, 4.7%).

On methylation with methanolic dimethyl sulphate and potassium carbonate, pinocembrin gave O,0-dimethyl-pinocembrin, m.p.  $156^\circ$ - $157^\circ$ .

##### 3.4.2.1.2. Alpinetin (E. sieberi).

Alpinetin had  $R_f$  0.87 in BAW, 0.04 in HOAc, 0.60 in CEF, and 0.62 in MCP. It crystallized from acetone in short white needles, m.p.  $218^\circ$ - $220^\circ$  (lit.<sup>69g</sup>  $223^\circ$ ), and had  $[\alpha]_D^{15} -46.0$  (c, 0.9 in  $CHCl_3$ );  $\lambda_{max}$  285, 322 (sh), nm,  $\log \epsilon_{max}$  4.37, 3.94;  $\lambda_{max}$  in MeOH/NaOAc:

321 nm;  $\delta$  2.78 ( $\underline{H}_{3eq}$ , q), 3.16 ( $\underline{H}_{3ax}$ , 1), 3.92 (MeO, s), 5.66 ( $\underline{H}_2$ , q) 6.16 ( $\underline{H}_6$ , d), 6.24 ( $\underline{H}_8$ , d), 7.63 (ring-B  $\underline{H}$ , m), 11.20 ( $-\underline{OH}_7$ , m, removed by  $D_2O$ );  $J_{2,3eq}$  3.2,  $J_{2,3ax}$  13.1,  $J_{3eq,3ax}$  17.0,  $J_{6,8}$  3.0. (Found: C, 71.1; H, 5.3. Calc. for  $C_{16}H_{14}O_4$ : C, 71.1; H, 5.2%).

On methylation with diazomethane in DMSO, alpinetin gave O,O-dimethylpinocembrin, m.p.  $155^\circ$ - $156^\circ$  undepressed on admixture with a synthetic sample.

#### 3.4.2.1.3. O,O-dimethylpinocembrin (E. sieberi).

Isolated as described above from E. sieberi extracts, (3) had  $R_f$  0.94 in BAW, 0.07 in HOAc, 0.75 in CEF, and 0.79 in MCP. It crystallized from acetone in long white needles, m.p.  $159^\circ$ - $160^\circ$  undepressed by admixture with a synthetic sample, and had  $[\alpha]_D^{15} -45.8^\circ$  (c, 2.0 in 50% MeOH/ $CHCl_3$ );  $\lambda_{max}$  283, 322 (sh) nm, log  $\epsilon_{max}$  4.39, 3.66;  $\delta(CDCl_3, TMS)$  2.77 ( $\underline{H}_{3eq}$ , q), 3.02 ( $\underline{H}_{3ax}$ , q), 3.74 (7-MeO, s), 3.89 (5-MeO, s), 5.40 ( $\underline{H}_2$ , q), 6.10 ( $\underline{H}_6$ , d), 6.18 ( $\underline{H}_8$ , d), 7.42 (ring-B  $\underline{H}$ , s)  $J_{2,3eq}$  3.2,  $J_{2,3ax}$  13.0,  $J_{3eq,3ax}$  17.0,  $J_{6,8}$  3.0.  $\delta(CDCl_3-C_6D_6)$ : + 0.43 (5-MeO), + 0.64 (7-MeO). (Found: C, 72.0; H, 5.6.  $C_{17}H_{16}O_4$  requires C, 71.8; H, 5.7%).

The major peaks of the mass spectra of all three of the above flavanones are combined in Chart 28, page 74 and the suggested fragmentation pattern in Chart 29, page 75.

### 3.4.2.2. Unknown D - Flavones (E. coccifera).

#### 3.4.2.2.1. Apigenin (4', 5,7-trihydroxyflavone).

Apigenin had  $R_f$  0.90 in BAW (415), 0.87 in TBA, 0.05 in 6HOAc and 0.08 in 15HOAc. It crystallized as yellow needles from ethanol, m.p.  $345^\circ$ - $346^\circ$  (lit.<sup>35f</sup>  $348^\circ$ - $350^\circ$ );  $\lambda_{\max}$  in MeOH: 266, 295 (sh), 335, in MeOH/NaOMe: 275, 325, 393 (no dec.), in MeOH/ $AlCl_3$ : 274, 300, 346, 385, in MeOH/ $AlCl_3$ +HCl: 275, 298, 338, 382, in MeOH/NaOAc: 274, 300, 375, in MeOH/NaOAc+ $H_3BO_3$ : 267, 302 (sh), 340 nm;  $\nu_{\max}$  3330, (broad), 3280, (broad), 1655 (chelated C=O), 1610, 1590, 1560, 835 (strong)  $cm^{-1}$ ;  $\delta$  (DMSO- $d_6$ , int. TMS), 6.31 (d, 1H,  $H_6$ ,  $J_{meta} = 3$  Hz), 6.67 (d, 1H,  $H_8$ ,  $J_{meta} = 3$  Hz, partly obscured), 6.88 (s, 1H,  $H_3$ ), 6.98 (d, 2H,  $H_{3',5'}$ ,  $J_{ortho} = 9$  Hz), 7.99 (d, 2H,  $H_{2',6'}$ ,  $J_{ortho} = 9$  Hz), 10.42 (broad m, 1H,  $OH_4$ , removed with  $D_2O$ ), 10.60 (broad m, 1H,  $OH_7$ , removed with  $D_2O$ ), 12.86 (s, 1H,  $OH_5$ , chelated, removed partially with  $D_2O$ ); mass spectrum m/e (rel. intensity) 270 (18), 153 (30), 152 (20), 121 (70), 118 (67) with suggested fragmentation in Chart 32, page 78.

#### 3.4.2.2.2. Sideroxylin (4',5-dihydroxy-7-methoxy-6,8-dimethylflavone).

Sideroxylin had  $R_f$  0.92 in BAW (612), 0.90 in BAW (415), 0.05 in 6HOAc, 0.07 in 15HOAc and 0.92 in PhOH. It crystallized from ethanol as yellow needles, m.p.  $285^\circ$ - $286^\circ$  (lit.<sup>55</sup>  $286^\circ$ - $287^\circ$ ), undepressed on admixture with an authentic sample;  $\lambda_{\max}$  in MeOH: 277, 295 (sh),

328, in MeOH/NaOMe: 276, 320, 390 (no dec.), in MeOH/  
 $\text{AlCl}_3$ : 282, 306, 351, in MeOH/ $\text{AlCl}_3 + \text{HCl}$ : 283, 306, 345,  
 in MeOH/NaOAc: 276, 295 (sh), 332, 385, in MeOH/NaOAc  
 $+ \text{H}_3\text{BO}_3$ : 276, 295 (sh), 327 nm;  $\nu_{\text{max}}$  3495, 1640 (chelated  
 $\text{C}=\text{O}$ ), 1600, 1570, 1540, 1250 (OMe), 1450, ( $\text{CH}_3$ ), 1375  
 ( $\text{CH}_3$ ), 1100, 840  $\text{cm}^{-1}$ ;  $\delta$  (DMSO- $\text{d}_6 + \text{CDCl}_3$ ), 2.21 (s, 3H,  
 $\text{CH}_3$ -6) 2.39 (s, 3H,  $\text{CH}_3$ -8), 3.81 (s, 3H,  $\text{OCH}_3$ -7), 6.59  
 (s, 1H,  $\text{H}_3$ ), 7.00 (d, 2H,  $\text{H}_{3',5'}$ ,  $J_{\text{ortho}} = 8.5 \text{ Hz}$ ), 7.80  
 (d, 2H,  $\text{H}_{2',6'}$ ,  $J_{\text{ortho}} = 8.5 \text{ Hz}$ ), 8.68 (broad m, 1H,  $\text{OH}_4$ ,  
 removed with  $\text{D}_2\text{O}$ ), 12.96 (s, 3H,  $\text{CH}_3$ -6), 2.38  
 (s, 3H,  $\text{CH}_3$ -8), 3.78 (s, 3H,  $\text{OCH}_3$ -7), 6.60 (s, 1H,  $\text{H}_3$ ),  
 7.03 (d, 2H,  $\text{H}_{3',5'}$ ,  $J_{\text{ortho}} = 8.5 \text{ Hz}$ ), 7.81 (s, 2H,  $\text{H}_{2',6'}$ ,  
 $J_{\text{ortho}} = 8.5 \text{ Hz}$ ), 8.75 (s, 1H,  $\text{OH}_4$ , removed with  $\text{D}_2\text{O}$ ),  
 13.00 (s, 1H,  $\text{OH}_5$ , partially removed with  $\text{D}_2\text{O}$ );  
 $\delta(\text{CDCl}_3 - \text{C}_6\text{D}_6) + 0.60$  ( $\text{OCH}_3$ -7); mass spectrum m/e (rel.  
 intensity) 312 (22), 297 (21), 284 (7), 194 (11), 193 (14),  
 179 (29), 164 (36), 151 (74), 142 (18), 136 (27), 121  
 (45), 118 (33), 105 (37) with fragmentation shown in  
Chart 34, page 80. (Found: C, 69.3; H, 5.3. Calc. for  
 $\text{C}_{18}\text{H}_{16}\text{O}_5$ : C, 69.2; H, 5.2%).

#### 3.4.2.2.3. 4',5-dihydroxy-7-methoxy-6-methylflavone.

It has not been possible to isolate sufficient of  
 this compound for tests other than  $R_f$  values and a u.v.  
 spectrum but some conclusions may be drawn from other  
 spectra of the mixture of this compound and sideroxylin.  
 (See discussion, pages 151-153). The compound had  $R_f$  (P.C.)

identical with sideroxylin, but a distinct  $R_f$  of 0.72 in BPF (t.l.c.);  $\lambda_{\max}$  in MeOH: 275, 295 (sh), 328. in MeOH/NaOMe: 270, 297 (sh), 386, in MeOH/ $AlCl_3$ : 282, 302, 350, 410 (sh), in MeOH/ $AlCl_3$ +HCl: 283, 302, 348, 410 (sh), in MeOH/NaOAc: 273, 295 (sh), 380, in MeOH/NaOAc+ $H_3BO_3$ : 275, 295 (sh), 328 nm; the integrations of the second C-methylflavone proton signals are given as " $\frac{1}{3}$ " to suggest the component is 25% of the mixture;  $\delta$  (DMSO- $d_6$ ), 2.40 (s,  $\frac{1}{3} \times 3H$ ,  $CH_3$ -6), 4.30 (s,  $\frac{1}{3} \times 3H$ ,  $OCH_3$ -7), 7.02 (s,  $\frac{1}{3} \times 1H$ ,  $H_8$ ), 7.06 (s,  $\frac{1}{3} \times 1H$ ,  $H_3$ ), 7.32 (d,  $\frac{1}{3} \times 2H$ ,  $H_{3',5'}$ ,  $J_{ortho} = 8.5$  Hz), 8.22 (d,  $\frac{1}{3} \times 2H$ ,  $H_{2',6'}$ ,  $J_{ortho} = 8.5$  Hz), 8.49 (m,  $\frac{1}{3} \times 1H$ ,  $OH_{4'}$ , removed with  $D_2O$ ), 13.40 (s,  $\frac{1}{3} \times 1H$ ,  $OH_5$ , partially removed with  $D_2O$ ); the trimethylsilyl ether gave  $\delta$  ( $CDCl_3$ ), 2.10 (s,  $\frac{1}{3} \times 1H$ ,  $CH_3$ -6), 3.92 (s,  $\frac{1}{3} \times 3H$ ,  $OCH_3$ -7), 6.50 (s,  $\frac{1}{3} \times 1H$ ,  $H_8$ ), 6.61 (s,  $\frac{1}{3} \times 1H$ ,  $H_3$ ), 6.97 (d,  $\frac{1}{3} \times 2H$ ,  $H_{3',5'}$ ,  $J_{ortho} = 8.5$  Hz), 7.72 (d,  $\frac{1}{3} \times 2H$ ,  $H_{2',6'}$ ,  $J_{ortho} = 8.5$  Hz); mass spectrum m/e (rel. intensity) 298 (24), 283 (9), and other peaks are shown in Chart 40, page 86, together with the fragmentation pattern in Chart 39, page 85.

#### 3.4.2.3. Unknown A (4-O-methylellagic acid) (E. sieberi).

This monomethoxyellagic acid had  $R_f$  0.28 in TBA, 0.46 in BAW (612), 0.38 in BAW (415), 0.15 in 6HOAc and 0.30 in 15HOAc. It crystallized as white needles from aqueous alcohol, m.p.  $286^\circ$ - $300^\circ$ ;  $\lambda_{\max}$  in MeOH: 255, 275 (sh), 312 (sh), 362, in MeOH/NaOMe: 242 (sh), 279, 292

(sh), 325 (sh), 383, in MeOH/AlCl<sub>3</sub>: 265, 305 (sh), 352 (sh), 372, in MeOH/AlCl<sub>3</sub>+HCl: 254, 300 (sh), 352 (sh), 363, in MeOH/NaOAc: 255, 275, 295 (sh), 368 (sh), 395 (sh), in MeOH/NaOAc+H<sub>3</sub>BO<sub>3</sub>: 260, 276 (sh), 312 (sh), 370 nm;  $\nu_{\max}$  3420, 3322, 3160 (bound OH's), 1720 (non-chelated C=O), 1610, 1576, 1288 (OCH<sub>3</sub> stretch), 1077 (OCH<sub>3</sub> stretch), 1236, 1206, 1106 (lactone C-O stretch), 1027 (lactone C-O stretch), 916, 886 (isolated ArH), 805 cm<sup>-1</sup>;  $\delta$ (DMSO-d<sub>6</sub>, ext. TMS), 8.11 (s, 1H, H<sub>5</sub>), 7.85 (s, 1H, H<sub>5</sub>), 5.40 (m, 3H, OH<sub>3,3',4'</sub> removed with D<sub>2</sub>O), 4.40 (s, 3H, OCH<sub>3</sub> 4): for the methyl ether  $\delta$ (CDCl<sub>3</sub>), 7.63 (s, 2H, H<sub>5,5'</sub>), 3.36 (s, c. 12H, OCH<sub>3</sub> 3,3',4,4'),  $\delta$ (C<sub>6</sub>D<sub>6</sub>), 7.65 (s, 2H, H<sub>5,5'</sub>), 2.93 (s, c. 12H, OCH<sub>3</sub> 3,3',4,4'),  $\delta$ (CDCl<sub>3</sub>-C<sub>6</sub>D<sub>6</sub>) = +0.43; mass spectrum m/e (rel. intensity) 316 (100), 301 (38), 273 (19), 245 (7), 218 (8), 189 (11), 161 (6), corresponding to the fragmentation shown in Chart 47, page 93. (Found: C, 56.7; H, 2.4. C<sub>15</sub>H<sub>8</sub>O<sub>8</sub> requires C, 56.9; H, 2.5%).

3.4.2.4. Unknown B (1,5-dimethyl-2,6-bis(3,4,5-trihydroxyphenyl) furo [1,5-c] furan).

This compound had R<sub>f</sub> 0.75 in BAW (612), 0.72 in BAW (415), 0.66 in TBA, 0.08 in 6HOAc and 0.15 in 15HOAc. It crystallized as off-white prisms from ethanol, m.p. 120°-122° and had  $[\alpha]_D^{15}$  79.1° (c.1.0 in CHCl<sub>3</sub>);  $\lambda_{\max}$  in MeOH: 235 (sh), 283, 370 (sh), in MeOH/NaOMe: 300 (broad) (dec.), in MeOH/AlCl<sub>3</sub>: 286, 310 (sh), 358 (sh), 375 (sh), 430 (sh), in MeOH/AlCl<sub>3</sub>+HCl: 286, 360, 375 (sh),

in MeOH/NaOAc: 287, 360, 380 (sh), in MeOH/NaOAc+H<sub>3</sub>BO<sub>3</sub>: 285, 360, 382 (sh),  $\nu_{\max}$  3410 (broad), 3130 (broad), 1720 (broad), 1600, 1510, 1416, 1280, 1220, 1130, 1110 cm<sup>-1</sup>;  $\delta$  (DMSO-d<sub>6</sub>, ext. TMS), 1.55 (s, 3H, CH<sub>3</sub>), 2.25 (s, 3H, CH<sub>3</sub>), 3.72 (m, 2H, furofuran H), 3.98 (m, 2H, furofuran H), 4.40 (m, 2H, furofuran H), 7.45 (m, 2H, ArH), 7.93 (m, 2H, ArH), 8.52 (m, 2H, ArOH, removed with D<sub>2</sub>O), 9.02 (m, 4H, ArOH, removed with D<sub>2</sub>O); mass spectrum m/e (rel. intensity) 390 (100), 304 (13), 302 (14), 287 (19), 285 (19), 255 (26), 254 (26), 206 (11), 204 (11), 157 (19) as in Chart 49, page 95.

3.4.2.5. Unknown F (a tetrahydroxydibenzofuran-dicarboxylic acid). (E. sieberi).

The compound had R<sub>f</sub> 0.47 in BAW (612), 0.51 in BAW (415), 0.37 in TBA, 0.12 in 6HOAc and 0.24 in 15HOAc. It crystallized from ethanol as yellow needles, m.p. 175°-180°;  $\lambda_{\max}$  in MeOH: 256, 305, 351 (sh), 366, in MeOH/NaOMe: 256 (sh), 283, 355 (sh), in MeOH/AlCl<sub>3</sub>: 241 (sh), 267, 320 (sh), 382 (sh), in MeOH/AlCl<sub>3</sub>+HCl: 256, 310 (sh), 352 (sh), 365, in MeOH/NaOAc: 258, 274, 360 (sh), in MeOH/NaOAc+H<sub>3</sub>BO<sub>3</sub>: 260, 305, 360 (sh), 375 nm;  $\nu_{\max}$  3370 (bonded OH), 3340 (carboxylic OH), 1712 (strong) (carboxylic C=O), 1605, 1520, 1280-1300 (acid), (1120, 1040, 1010) (C-O stretch), 925, 888 (isolated ArH), 868 cm<sup>-1</sup>;  $\delta$  (DMSO-d<sub>6</sub>, ext. TMS), 7.94 (s, 2H, ArH), 10.30 centre (broad m, 6H, ArOH, ArCOOH,

removed with  $D_2O$ ); mass spectrum  $m/e$  (rel. intensity)  
320 (10), 304 (6), 276 (100), 232 (10), 200 (5),  
176 (70), 177 (20), 150 (100), 131 (60) as in Chart 53,  
page 99.

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CHAPTER 4.

IDENTIFICATION - DISCUSSION.

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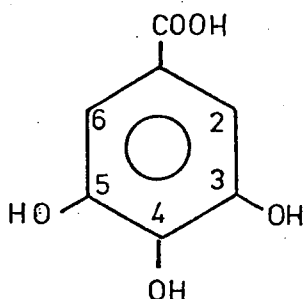
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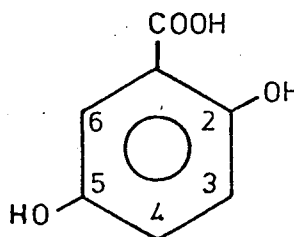
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CHAPTER 4.4.0. POLYPHENOLS OF KNOWN STRUCTURE.

The evidence for the identification of the polyphenols of known structure isolated in this work is now discussed.

4.0.1. HYDROXYBENZOIC ACIDS.

( 2 )



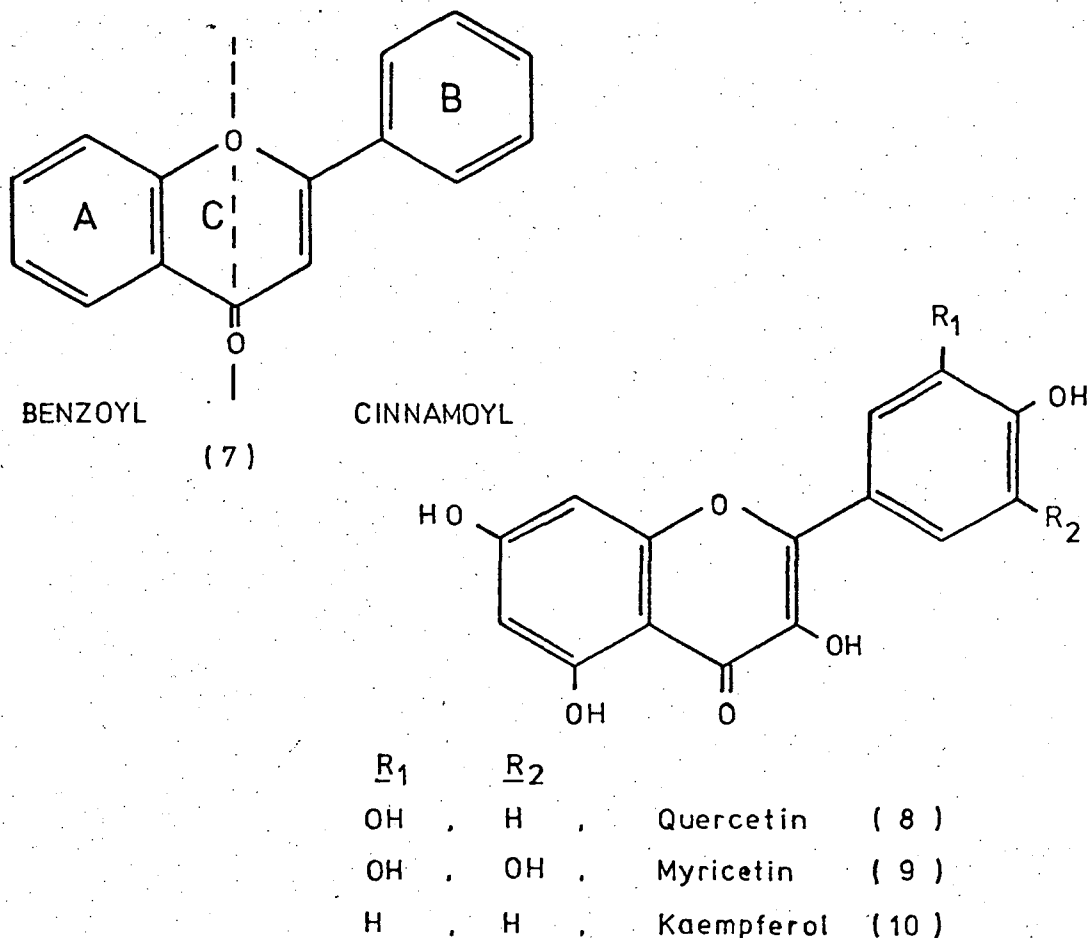
( 3 )

Gallic (2) and gentisic (3) acids were readily identified from their n.m.r., and mass spectra<sup>81</sup> with chromatographic behaviour corresponding to that recorded by Hillis.<sup>48</sup> The melting point of each agreed with literature values.<sup>69b,69c</sup>

The identity of protocatechuic acid (1, page 12) seems certain from comparison of its n.m.r. spectrum with those of the above acids. (Chart 10, page 54 ). In combination with the mass spectra shown in (Chart 11, page 55 ), the u.v. spectral shift obtained with boric acid in acetate<sup>64</sup> and a chromatogram showing separate spots for the two dihydroxyacids, together with a

melting point in agreement with the literature,<sup>69a</sup>  
completed the identification.

#### 4.0.2. FLAVONOLS.



The flavonols quercetin (8), myricetin (9) and kaempferol (10) have been isolated and the evidence for their identity is now given.

On paper chromatograms all three compounds had the yellow colour characteristic of flavonols with a free 3-hydroxyl<sup>12</sup> when viewed under u.v. light and their u.v. spectra showed the two bands typical of the

conjugated benzol-cinnamoyl system<sup>35a</sup> (7).

#### 4.0.2.1. Quercetin.

The shifts induced with sodium acetate,<sup>68</sup> acidified aluminium chloride<sup>64</sup> and boric acid in acetate<sup>64</sup> in u.v. spectra of quercetin showed its hydroxyl substitution which was confirmed by the n.m.r. spectrum. (Chart 12, page 56 ). Co-chromatography and melting point of the mixture with an authentic sample without depression, together with a satisfactory elemental analysis completed the identification of quercetin.

#### 4.0.2.2. Myricetin.

Myricetin was identified first from its n.m.r. spectrum which, when compared with that of quercetin (Chart 13, page 57), showed that the quercetin 5'-proton is no longer present in myricetin; in addition the myricetin spectrum shows a two-proton singlet for the protons in positions-2',6'.

Identification of myricetin was completed by comparison of its mass spectrum and proposed fragmentation with those of quercetin (Charts 14,15 pages 58,59 ) and finally by a mixed melting point without depression using an authentic sample, together with a satisfactory elemental analysis.

#### 4.0.2.3. Kaempferol.

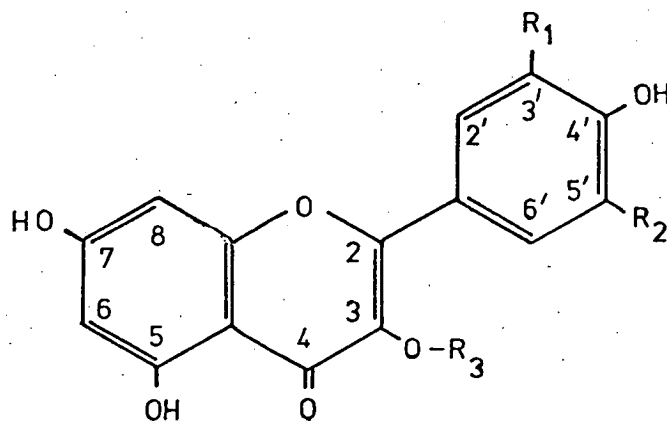
It is noteworthy that Hillis found chromatographically that E. coccifera was the only

peppermint to contain kaempferol<sup>49</sup> (10).

The presence of kaempferol in E. coccifera has been verified in this work by the isolation of the aglycone as well as by the oxidation of afzelin.

Spectral evidence, especially the n.m.r. signals of ortho-protons in positions-3', 5' and positions-2', 6' and meta-protons in positions-6,8 together with the chromatographic comparison with an authentic sample and melting point determination seemed unambiguous evidence for the presence of kaempferol.

#### 4.0.3. FLAVONOL GLYCOSIDES.



$R_1, R_2 = H, R_3 = \text{Rhamnosyl}$  : Afzelin

$R_1 = OH, R_2 = H, R_3 = \text{Rhamnosylglucosyl}$  : Rutin

$R_3 = \text{Rhamnosyl}$  : Quercitrin

$R_3 = \text{Glucosyl}$  : Isoquercitrin

$R_3 = \text{Galactosyl}$  : Hyperin

$R_1, R_2 = OH, R_3 = \text{Glucosyl}$  : Cannabiscitrin

#### 4.0.3.1. Afzelin (kaempferol-3-rhamnoside).

Peroxide oxidation<sup>22</sup> of this glycoside isolated from E. coccifera released the sugar as shown by paper chromatography. Chandler and Harper<sup>22</sup> found that controlled oxidation of glycosides with peroxide released only sugar residues from the 3-position and proved more reliable for glycoside differentiation than the standard method of exhaustive methylation applied by King and Acheson<sup>71</sup> and adapted by Nordström to the microscale.<sup>83</sup>

The products of the oxidation were identified by their  $R_f$  values in several solvents using the chromogenic spray of diazotised-p-nitroaniline<sup>102</sup> for kaempferol and p-anisidine hydrochloride<sup>75b</sup> for rhamnose.

The oxidation evidence was supported by u.v. spectral analysis of the glycoside, showing the presence of a free 7-hydroxyl by a bathochromic shift of the shorter wavelength band in sodium acetate.<sup>68</sup> The spectrum of the glycoside with sodium methoxide reagent showed no decomposition when run again some ten minutes after basification, confirming the absence of an alkali sensitive grouping of hydroxyls, such as a 3,3',4'-group.<sup>30</sup>

The similarity between the u.v. spectra run in aluminium chloride and acidified aluminium chloride showed the shift was due to a chelating 5-hydroxyl,



with no acid-labile complexes being formed with ortho-di-hydroxyls.<sup>37,103</sup>

The glycoside was thus identified as afzelin, kaempferol-3-rhamnoside, with the reported melting point.<sup>71</sup>

#### 4.0.3.2. Rutin (quercetin-3-rhamnosylglucoside).

Among the flavonol glycosides isolated from E. delegatensis,<sup>16a</sup> rutin was identified by comparison of its observed and published u.v.<sup>35b</sup> and n.m.r. spectral data, the latter for both rutin<sup>14</sup> and its trimethylsilyl ether.<sup>75g</sup> After acid hydrolysis, co-chromatography and mixed melting point without depression of a mixture with an authentic sample of quercetin confirmed the identify of the aglycone.

The sugar residues after hydrolysis were co-chromatographed with known sugars in two solvents and their  $R_f$  values showed them to be rhamnose and glucose and the identity of the diglycoside as quercetin-3-rhamnosylglucoside.

#### 4.0.3.3. Quercitrin (quercetin-3-rhamnoside) and isoquercitrin (quercetin-3-glucoside).

The other two glycosides isolated from E. delegatensis appeared from  $R_f$  values to monoglycosides<sup>76</sup> and peroxide oxidation, according to the method of Chandler and Harper,<sup>22</sup> gave in both cases the aglycone quercetin identified by comparison with an authentic sample. After oxidation one glycoside

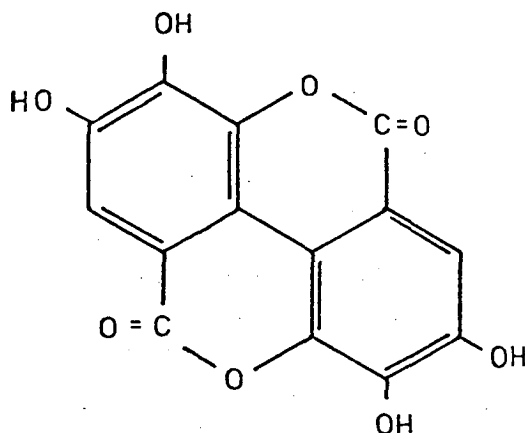
yielded rhamnose, the other glucose both identified by co-chromatography with authentic sugars.

The monoglycosides were therefore quercitrin (8), (quercetin-3-rhamnoside), its identity confirmed by comparing it with a known sample, and isoquercitrin (9), (quercetin-3-glucoside).

4.0.3.4. Hyperin (quercetin-3-galactoside) and Cannabiscitrin (myricetin-3-glucose).

The peroxide oxidation of the glycosides isolated from E. sieberi by the method of Chandler and Harper<sup>22</sup> determined that the sugar residue had been located in the 3-position. The aglycones released in the oxidation were identified as quercetin and myricetin by direct comparison of their properties with those of authentic samples. The sugars were identified as galactose and glucose respectively by chromatographic comparison with authentic sugars.

The glycosides were thus identified as hyperin (quercetin-3-galactoside) and cannabiscitrin (myricetin-3-glucose).

4.0.4. ELLAGIC ACID.

( 11 )

The final compound isolated from E. delegatensis was identified as ellagic acid (11) from its  $R_f$  values and characteristic chromatographic colours, being violet-blue (u.v. light, 365 nm) and dull yellow when fumed with ammonia.<sup>53b</sup> When sprayed with ferric ferricyanide and diazotised p-nitroaniline, paper chromatograms of the compound showed blue and yellow-brown respectively.<sup>53b</sup> Co-chromatography with an authentic sample showed no separation of spots.

It was necessary to examine the spectral properties of ellagic acid in some detail because two of Hillis' unknowns, (viz. A,B),<sup>48</sup> were provisionally identified by him as "ellagic acid-like compounds".

In methanol, ellagic acid gave a characteristic

u.v. spectrum<sup>65</sup> with a minor peak at 366 nm and an intense peak at 255 nm.

Particular note was taken of two u.v. spectral shifts induced by diagnostic reagents; the first, a hypsochromic shift of the long wavelength band when a methanolic solution of ellagic acid was treated with fused sodium acetate; secondly, a bathochromic shift of the short wavelength band upon treatment of a methanolic solution of the compound with sodium methoxide.

Comparison between these spectral shifts for ellagic acid and similar shifts in the u.v. spectra of unknown A proved valuable in the identification of the latter.<sup>97</sup> (cf. page 159, Table 4).

The i.r. spectrum of ellagic acid showed a strong lactonic<sup>107b</sup> non-chelated carbonyl absorption at  $1720\text{ cm}^{-1}$ , with hydroxyl bands both in the region associated with inter-molecular hydrogen bonding ( $3600 - 3500\text{ cm}^{-1}$ ) and in the region associated with intra-molecular hydrogen bonding<sup>107a</sup> ( $3300\text{ cm}^{-1}$ ).

Chart 16 (page 60) gives the mass spectrum of ellagic acid with the fragmentation suggested by Nelson and Porter.<sup>81</sup>

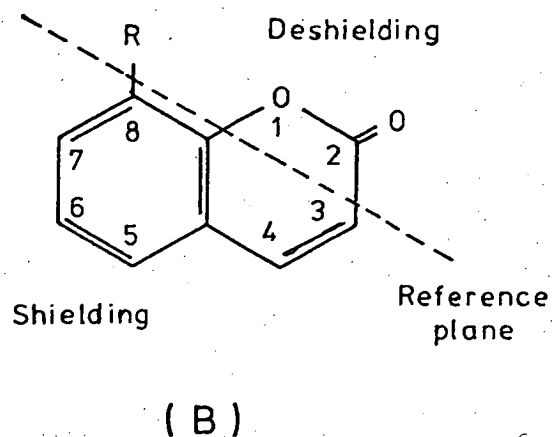
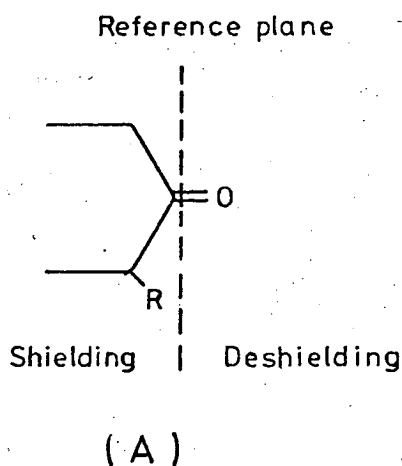
Ellagic acid proved sufficiently soluble in deuteropyridine at  $80^{\circ}\text{C}$  to obtain the n.m.r. Chart 17, page 61, which gives a two-proton singlet at  $\delta\ 8.01$ ,

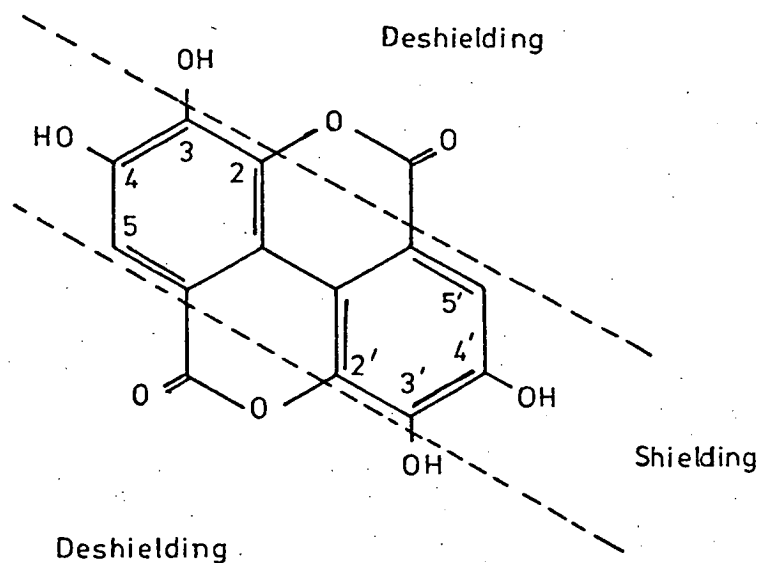
and doublets at  $\delta 5.08$  and  $\delta 4.25$ , although retaining signals due to solvent of crystallization.

The singlet signal due to the 5,5'-protons of ellagic acid is confirmed by the n.m.r. spectrum of its trimethylsilyl ether (Chart 18, page 62) with the two-proton signal at  $\delta 7.63$  p.p.m.

While no work has been published on the n.m.r. spectra of ellagic acid-type dilactones, work on ketones (A)<sup>38</sup> and coumarins (B)<sup>38</sup> seems to allow inferences to be drawn for the dilactones.

Use is made of these inferences in the identification of unknown A, page 154, so further discussion is apposite.





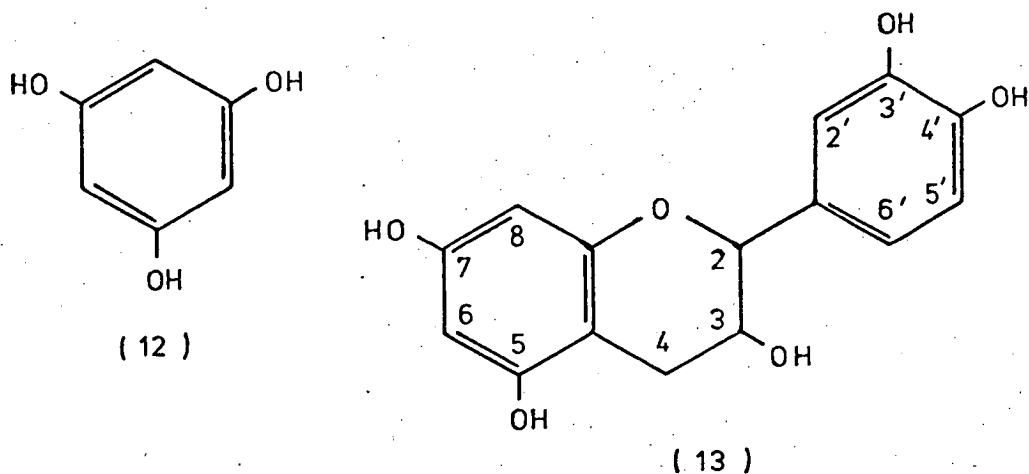
( C )

Previous work on ketones<sup>38</sup> (A) showed that a reference plane could be drawn through the carbonyl carbon atom and perpendicular to the plane of the molecule such that protons in front of the plane were deshielded while those behind the plane were shielded.

In coumarins (B)<sup>38</sup> a similar reference plane was found to occur but with the plane shifted slightly by the heterocyclic ring, with maximum shielding being shown by substituents at C-4 and minimum shielding by substituents at C-8.

By analogy it is proposed in this work that similar planes can be drawn for ellagic acid type compounds (C). The hydroxyls that would incur the maximum shielding would be those resonating at  $\delta 4.25$  p.p.m., namely the 4,4'-hydroxyls, while the hydroxyls incurring the minimum shielding would be those at positions-3,3', resonating at  $\delta 5.08$  p.p.m.

4.0.5. (+)-CATECHIN.



A compound isolated from a Sephadex column gave a red colour on a paper chromatogram when sprayed with methanolic vanillin-hydrochloric acid,<sup>13</sup> a yellow colour when sprayed with ethanolic *p*-toluene sulphonic acid,<sup>96</sup> reactions given only by flavans containing an A-ring derived from phloroglucinol (12) i.e. a 5,7-dihydroxyflavan.

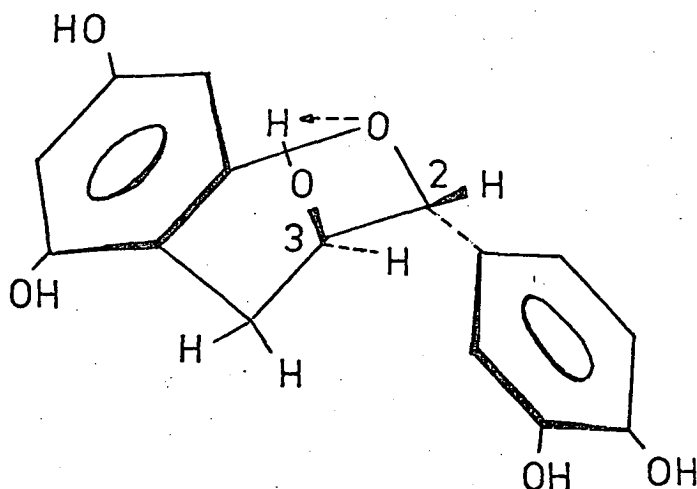
Changes in the u.v. spectrum, re-run

after some minutes, when a methanolic solution of the compound was treated with sodium methoxide, indicated the presence of an alkali sensitive group of hydroxyls such as a 3,3',4'-group.<sup>30</sup> The existence of the 3',4'-dihydroxyl group being further supported by the bathochromic shift of the cinnamoyl band of the u.v. spectrum of a methanolic solution of the compound treated with boric acid and sodium acetate.<sup>75d</sup>

The n.m.r. spectrum of the flavan (Chart 19, page 63 ) run in deuterodimethylsulphoxide to show the aromatic proton signals indicated that the flavan was an isomer of 3',4',5,7-tetrahydroxyflavan-3-ol (13). An additional spectrum (Chart 20, page 64 ) run in deuteropyridine gave signals for the methylene protons at C-4 and the methine protons at C-3 and C-4 which were analysable as a first-order ABMX system.<sup>26</sup>

The size of the coupling between the C-2, C-3 protons (8 Hz)<sup>26</sup> showed them to be trans- and thus the isolated compound to be a catechin rather than an epicatechin. The specific rotation was measured as positive, similar to that of an authentic sample, and the catechin was identified as the (+)-enantiomer of the catechins (Figure 6, overleaf).



(+)- CATECHIN2R : 3SFigure 6.

The mass spectrum of the flavan showing a molecular ion of 290 and a base peak due to a typical o-hydroxybenzyl cation<sup>24</sup> at 139 supported the above identification and as Chart 21, page 65 illustrates, the major peaks correspond to a retro-Diels-Alder fission of the heterocyclic ring<sup>4</sup> accompanied by hydrogen transfer mainly from the 3-hydroxyl.<sup>87</sup>

The above evidence was supported by an i.r. spectrum which showed strong hydroxyl absorption indicative of both inter-molecular and intra-molecular hydrogen bonding but no absorption attributable to a carbonyl group.

A melting point in agreement with the literature

and undepressed on admixture with an authentic sample, together with a satisfactory elemental analysis completed the identification of the flavan as (+)-catechin or 3', 4', 5,7-tetrahydroxy-2,3-trans-flavan-3-ol.

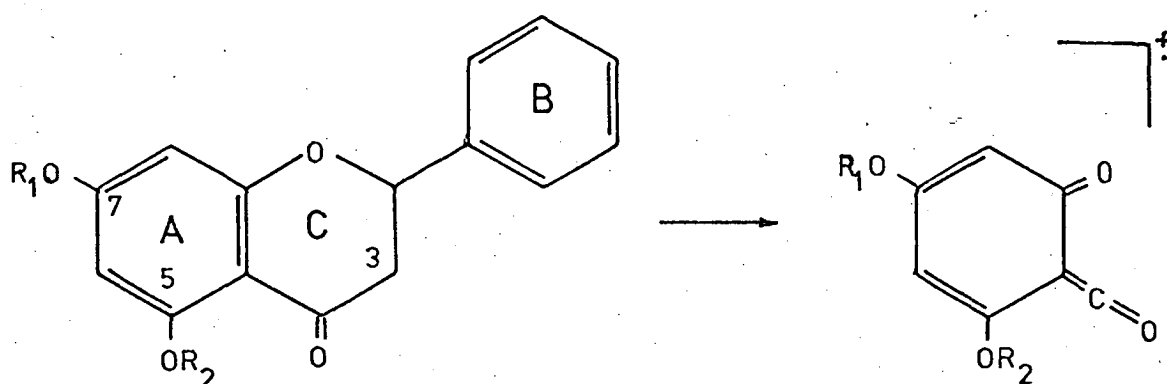
#### 4.1. POLYPHENOLS OF UNKNOWN STRUCTURE.

##### 4.1.1. UNKNOWN D - FLAVANONES.<sup>15</sup>

From a leaf extract of E. sieberi (page 27) were isolated three closely related substances with chromatographic behaviour similar to compound D, which have properties corresponding to flavanones.

In particular, all three compounds showed a dull red colour when sprayed with sodium borohydride followed by exposure to concentrated hydrochloric acid fumes.<sup>39</sup> The i.r. spectra showed the presence of carbonyl groups, confirmed by reaction with dinitrophenylhydrazine; the u.v. spectra, with strong peaks around 286 nm, were consistent with those of flavanones, and likewise the mass spectra,<sup>9</sup> which furthermore had strong M-77 ions indicating unsubstituted B. rings.

The latter inference was supported by five-proton multiplets in the aromatic region of the n.m.r. spectra, and by characteristic i.r. peaks around 765 and 700  $\text{cm}^{-1}$ .<sup>19</sup>



$\underline{R}_1$	$\underline{R}_2$	
H	H	: (14)
H	Me	: (15)
Me	Me	: (16)
Me	H	: (16a)

M - 104

(16b)

All three compounds were reduced by strong acid and magnesium but not zinc: this indicated they had no 3-hydroxyl groups,<sup>100</sup> the absence of which was confirmed by the M-104 ions (16b) which formed the base peaks in their mass spectra.

The n.m.r. spectra of all three flavanones are of the ABX type with average values of the coupling constants  $J_{AX} = 13.0$  Hz and  $J_{BX} = 3.0$  Hz. It is known from studies of cyclohexane derivatives and analogous compounds<sup>92</sup> that two hydrogen atoms carried by adjoining carbon atoms, with one hydrogen equatorial and the other axial give coupling constants of 2-5 Hz

whereas when both hydrogens are axial the coupling constant is larger (6-11 Hz).

In the case of the flavanones examined here the coupling constant of 13.0 Hz is so high that it can only represent an axial-axial coupling so that H-2 would be axial and the 2-phenyl equatorial or vice versa.

Further, assuming the Karplus formula,<sup>27</sup> modified by Williamson and Johnson<sup>108</sup> predicting a relation between the dihedral angle ( $\phi$ ) and the vicinal coupling constant, is always true for flavanones,<sup>79</sup> it is possible to calculate the dihedral angles which the two C-3 hydrogen atoms make with the C-2 hydrogen:

$$J_{H_2 - H_{3a}} = 13.0 = 16 \cos^2 \phi = 154^\circ \text{ (nearest degree)}$$

$$J_{H_2 - H_{3e}} = 3.0 = 10 \cos^2 \phi = 57^\circ \text{ (nearest degree)}$$

Chart 22, page 68, shows the application of n.m.r. spectral data to determine relative configuration between C-2 and C-3 hydrogen atoms in flavanones.

Each of the flavanones was found to be laevo-rotatory with the one optical centre at C-2; hence they belong to the same configurational series.<sup>1</sup>

Chart 23, page 69, shows a comparison of the important portions of the infra-red spectra of the three flavanones and Chart 24, page 70, summarises the n.m.r. spectral signals of Charts 25, 26, 27, pp. 71, 72, 73.

with their assignments.

#### 4.1.1.1. Pinocembrin.

The compound of lowest molecular weight, which was present in least amount, reacted with ferric chloride and coupled with diazotized p-nitroaniline.<sup>48</sup>

Its u.v. spectrum showed large bathochromic shifts with aluminium chloride<sup>66</sup> and with sodium acetate,<sup>66</sup> indicating the presence of hydroxyl groups at positions 5 and 7 respectively. The broad i.r. band around  $3120\text{ cm}^{-1}$  and the carbonyl absorption at  $1629\text{ cm}^{-1}$  supported the presence of a 5-hydroxyl group, and the data indicated that the compound was pinocembrin (14).

Its identity was confirmed by a comparison of its properties with those of a synthetic specimen.

#### 4.1.1.2. Alpinetin.

The second flavanone also gave a ferric chloride test, but coupled slowly with diazotized p-nitroaniline.<sup>48</sup> Its u.v. spectrum showed a bathochromic shift with sodium acetate<sup>66</sup> but not with aluminium chloride,<sup>100</sup> indicating that the compound had a 7-hydroxyl but no 5-hydroxyl group. This was consistent with the presence of a methoxyl proton resonance in the n.m.r. spectrum, and with an i.r. hydroxyl absorption at  $3475\text{ cm}^{-1}$  and a carbonyl at  $1660\text{ cm}^{-1}$ .

The substance thus has the structure of alpinetin<sup>95</sup> (15) a rather rare flavanone not previously encountered in eucalypts.

Its melting point agreed with that reported for alpinetin, but was over  $80^{\circ}$  higher than that of the isomeric pinostrobin<sup>69g</sup>(16a); moreover, an authentic sample of the latter showed a u.v. shift with aluminium chloride but not with sodium acetate.

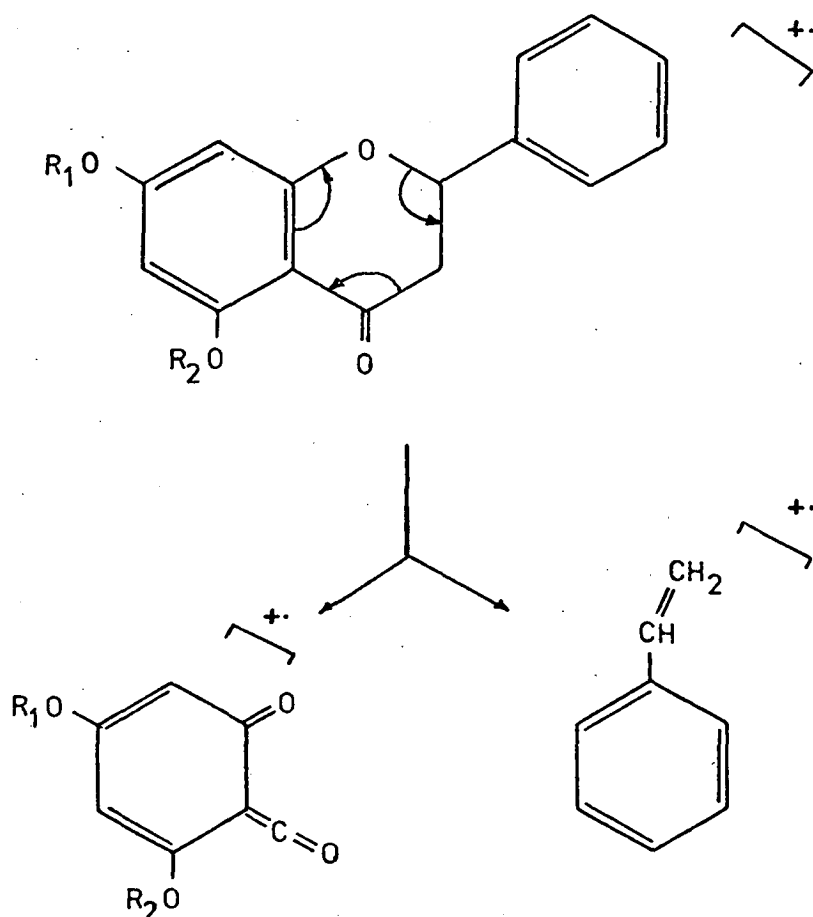
The identity was confirmed by conversion into its O-methyl derivative, which proved identical with the O,O-dimethyl derivative prepared from pinocembrin.

4.1.1.3. O,O-dimethylpinocembrin.

The third flavanone gave none of the phenolic tests or u.v. shifts described for (14) or (15), but it had an i.r. carbonyl absorption at  $1671\text{ cm}^{-1}$ , and its n.m.r. spectrum showed two methoxyl proton resonances with benzene shifts corresponding to locations at positions 5 and 7.<sup>109</sup>

Its identity as O,O-dimethylpinocembrin (16), which does not appear to have been found previously in nature, was confirmed by a comparison of its properties with those of an authentic sample prepared from pinocembrin.

Mass spectral fragmentation of the flavanones followed a pattern shown by Audier<sup>4</sup> to be a retro-Diels-Alder fission, illustrated below:



A fuller interpretation of the mass spectra of the flavanones and assignment of peaks is shown in Charts 28,29 (pages 74,75 ).

It should be noted for chemotaxonomy that, using similar evidence, a compound identified as pinocembrin (14) was isolated from E. delegatensis.

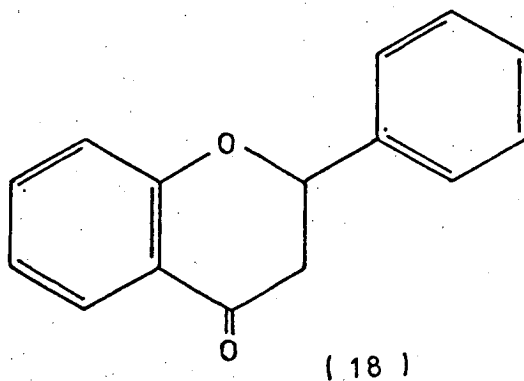
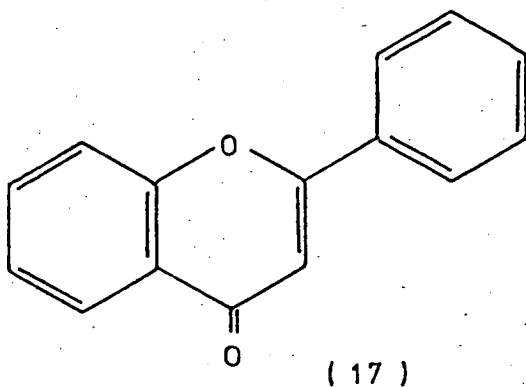
#### 4.1.2. UNKNOWN D - FLAVONES.

As explained in Chapter 2, page 42, a three component mixture of compounds was isolated from E. coccifera with chromatographic behaviour similar to Hillis' unknown D.<sup>48</sup>

Only two of these components were isolated in the pure state in sufficient quantity for complete spectral analysis. The amount isolated of the third component was enough only for chemical reactions in solution and u.v. spectral analyses.

None of these E. coccifera unknown D compounds gave a red or magenta colour on reduction with sodium borohydride and subsequent exposure to acid vapour,<sup>39</sup> as the E. sieberi flavanones had done.<sup>90</sup>

The evidence discussed here identified the unknown D compounds in E. coccifera as flavones (17) not flavanones (18). So that unknown D appears to be different mixtures of compounds in the two species from which it has been isolated in this work.





Evidence structurally significant for all components is given first followed by evidence peculiar to each.

All three compounds gave positive ferric chloride tests but inconclusive phenylhydrazine reactions: this relative unreactivity of the carbonyl group in flavones is reported by Gripenberg<sup>35e</sup> and Baker et al.<sup>6</sup>

The i.r. spectra confirmed the presence of carbonyl groups in the major components.

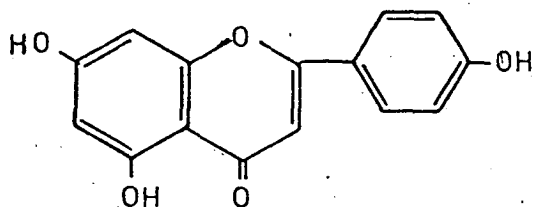
The u.v. spectra of all three components showed some similar features:: first, strong bands around 275 nm and 325 nm, consistent with a flavone conjugated benzoyl-cinnamoyl system;<sup>35a</sup> secondly, bathochromic shifts of the longer wavelength band upon addition of sodium acetate, in accord with the presence of a 4'-hydroxyl in ring B;<sup>35a</sup> thirdly, bathochromic shifts with aluminium chloride unaffected by addition of hydrochloric acid,<sup>67</sup> indicating the presence of a chelated 5-hydroxyl but no adjacent hydroxyls capable of labile complexes; fourthly, the lack of adjacent hydroxyls was confirmed by the failure of the methanolic spectra to shift with the boric acid/acetate reagent.<sup>64</sup>

The i.r. spectra of the major components supported the inference of a 4'-hydroxyl substitution in the B-ring by the presence of a strong peak around  $840\text{ cm}^{-1}$ ,<sup>74</sup> together with hydroxyl absorption around

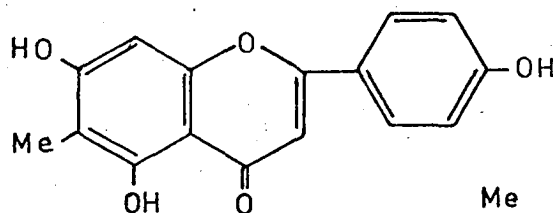
3330  $\text{cm}^{-1}$  found by Briggs<sup>19</sup> to be common among flavones with 4'-hydroxyl groups.

The n.m.r. spectra of the major components were similar: first, two doublets ( $J = 9 \text{ Hz}$ ) consistent with two pairs of ortho- protons in a para-substituted benzene, confirming the 4'-hydroxyl group; secondly, a sharp singlet at low field ( $\delta 6.6 - \delta 6.8 \text{ p.p.m.}$ ) was typical of the 3-proton of flavones.<sup>109</sup>

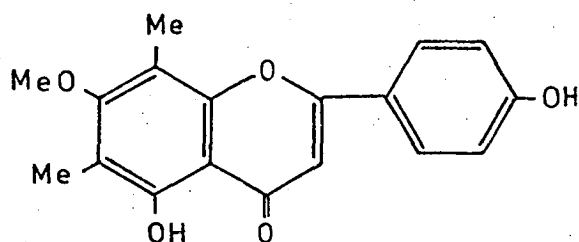
The 4'-hydroxyl pattern of the B-ring was also indicated by the mass spectra of the compounds which showed peaks corresponding to M-93 ions. Peaks at M-118 supported the n.m.r. evidence for the absence of 3-hydroxyls.



( 19 )



( 20 )



( 21 )

#### 4.1.2.1. Apigenin.

Considering each flavone in particular, the spot of lowest  $R_f$  corresponded to a compound whose u.v. spectrum showed, in addition to the above shifts, a bathochromic shift of the shorter wavelength band upon addition of sodium acetate, indicating the presence of a 7-hydroxyl group.<sup>68</sup>

This inference was supported by hydroxyl absorption around  $3280\text{ cm}^{-1}$  in the i.r. spectrum.<sup>19</sup>

The n.m.r. spectrum of this compound showed peaks assignable to meta-coupled 6,8-protons ( $J = 3\text{ Hz}$ ) in addition to the signals already discussed.

(Chart 30, page 76).

Chart 31, page 77, shows a molecular ion of 270 and the fragmentation coming from the retro-Diels-Alder fission of the heterocyclic ring<sup>4</sup> is suggested

in Chart 32, page 78 .

The melting point compared favourably with the literature<sup>35f</sup> and completed the identification of the compound as apigenin, (19), 4',5,7-trihydroxyflavone.

The mixture of higher  $R_f$  than that of apigenin appeared to be a mixture of C-methylflavones, considered to be comparatively rare plant extractives.<sup>53a,55</sup>

Although Hillis did not record C-methylflavones in his earlier survey he has since discovered their presence in eucalypts.<sup>55</sup>

One component of the mixture was isolated in the pure state and its identity is discussed first.

The second component was isolated in sufficient quantity for u.v. spectral analysis but further evidence for its identity is discussed by reference to the n.m.r. and mass spectra of the mixture of the C-methylflavones.

#### 4.1.2.2. Sideroxylin.

Unlike the u.v. spectrum of apigenin, that of the isolated C-methylflavone showed no bathochromic shift of the shorter wavelength band with sodium acetate, indicating the absence of a 7-hydroxyl in ring A.<sup>68</sup>

The n.m.r. spectrum revealed the presence of methyl groups at positions -6,8. It is characteristic of these that the 6-methyl signal is upfield from the 8-methyl.<sup>78</sup> The n.m.r. spectrum also showed a 7-methoxyl group shifted characteristically (+0.60 p.p.m.)

when a spectrum was run in benzene.<sup>109</sup>

Charts 33,34, pages 79,80, give the mass spectrum and a suggested fragmentation of the major C-methylflavone. This evidence, together with an undepressed melting point on admixture of an authentic sample, confirmed the identity of the compound as sideroxylin (21), 4',5-dihydroxy-7-methoxy-6,8-dimethylflavone, reportedly found only once before in nature.<sup>55</sup>

4.1.2.3. 4',5-dihydroxy-7-methoxy-6-methylflavone.

The second component of the mixture of components of higher  $R_f$  than that of apigenin appears also to be a C-methylflavone although the evidence for its identity is given tentatively.

The u.v. spectral shifts for this compound were identical with those of sideroxylin, suggesting a similar chromophore system and hydroxyl pattern.

When the n.m.r. spectra of pure sideroxylin and impure sideroxylin were compared and the only signal to disappear on purification was noted with its integration, it seemed to be in a position consistent with a 6-methyl group.<sup>78</sup> (Charts 35,36,37, pages 81,82,83).

The lone 8-proton signal which might be expected to appear in a compound without the 8-methyl of sideroxylin has been shown to appear in the same spectral region as the 3-proton,<sup>75f</sup> in compounds of similar substitution.<sup>78</sup>

A similar comparison of the n.m.r. spectra of sideroxylin and the trimethylsilyl ether of the sideroxylin mixture also showed, upon purification of the sideroxylin, the disappearance of only one signal, which was assignable to a 6-methyl group. (Charts 35,36,38 pages 80,81,84).

The i.r. spectra of pure sideroxylin and the mixture were identical, suggesting only a minor structural difference between the two compounds in the mixture.

The mass spectra of flavonoids have been reported recently. Reed and Wilson,<sup>94</sup> and Audier<sup>4</sup> made separate studies of the fragmentation of flavones but there has been no detailed study of the mass spectra of C-methylflavones.

From a study of the mechanism of fission of ring C, the heterocyclic ring, these workers have concluded that the major fragmentation mode in flavones is a retro-Diels-Alder fission as illustrated in Chart 32, page 78.

Audier<sup>4</sup> found that the nature of the substituents had a great influence on the relative intensities of the peaks which resulted from Diels-Alder rupture and that substituents displace the peaks of flavone itself as a function of their mass.

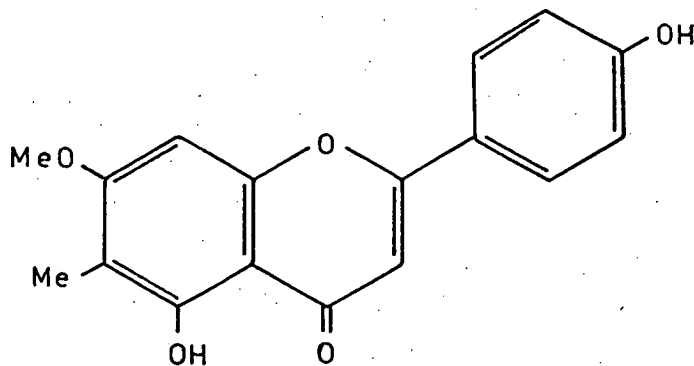
By analogy, Chart 34, page 80, suggests a mass fragmentation of sideroxylin corresponding to major

peaks of its mass spectrum in Chart 33, page 79 .

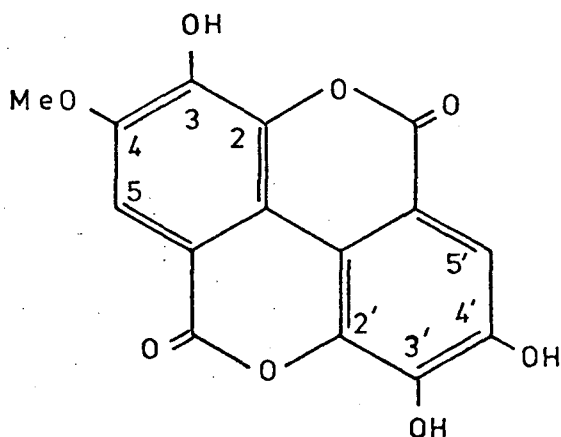
Chart 39, page 85 , suggests a similar mass fragmentation for the second C-methylflavone indicated by the peaks 298 ( $M^+$ ), 283 ( $M-15$ ) and  $135(\frac{M-28}{2})$  which seem to arise mainly from the impurity in Chart 40, page 86 , which shows the mass spectrum of the mixture.

These peaks could be explained by the C-methylflavone structure (20) proposed also on n.m.r. spectral evidence.

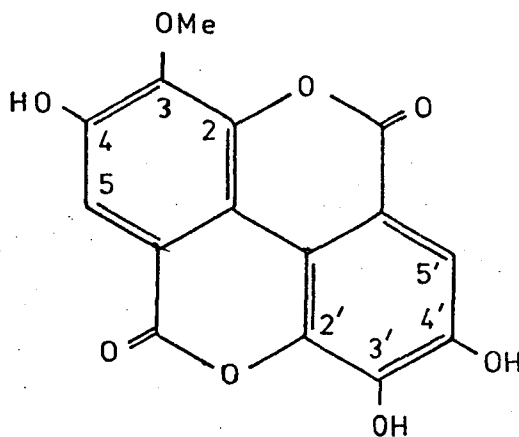
While isolation of this second component in the pure state has not been achieved, except enough for u.v. spectral analysis, evidence from n.m.r., i.r. and mass spectra of the mixture suggests the provisional identity of this component as 4',5-dihydroxy-7-methoxy-6-methylflavone (20), a compound not reported in the literature.



( 20 )

4.1.3. UNKNOWN A (4-O-methylellagic acid).

( 22 )



( 23 )

In this work the compound isolated with chromatographic behaviour similar to Hillis' unknown A<sup>48</sup> has properties corresponding to a monomethyl derivative of ellagic acid, a compound that seems not to have been found previously in nature.

The n.m.r. spectrum of unknown A showed a three-proton methoxyl singlet at  $\delta$  4.40 p.p.m., the downfield shift being due in part to the use of deuterodimethylsulphoxide with tetramethylsilane as external standard. In contrast to the two-proton



singlet for the aromatic protons of ellagic acid, the n.m.r. spectrum of the monomethyl derivative shows one proton singlet at  $\delta 7.85$  and  $\delta 8.11$  p.p.m. (Chart 41, page 87).

The separate n.m.r. signals for the 5,5'-protons in unknown A showed the difference in environment of each. Although data for analogous compounds is not reported in the literature, the magnitude of the shift difference for these signals (0.25 p.p.m.) suggested the environment difference was due to ortho- rather than meta- groups, <sup>107c</sup> favouring structure (22) for unknown A rather than structure (23).

Chemical shifts found by Williams <sup>107c</sup> for mono-substituted benzene protons relative to benzene are upfield by 0.23 p.p.m. for an o-OCH<sub>3</sub> and upfield by 0.37 p.p.m. for an o-OH, a shift difference of 0.16 p.p.m. These figures suggest the assignment of the signal at  $\delta 7.85$  p.p.m. to the proton ortho- to the 4'-hydroxyl and the signal at  $\delta 8.11$  p.p.m. to the proton ortho- to the 4-methoxyl in structure (22).

Further, the hydroxyl multiplet in the n.m.r. spectrum is in the region (  $\delta 5.00$  p.p.m.) previously assigned to 3,3'-hydroxyls in the discussion of ellagic acid on page 136 , supporting the deduction that 3,3'-hydroxyls are present in unknown A, i.e. structure (22).

In an attempt to secure unambiguous evidence for the location of the methoxyl in unknown A, the

compound was methylated and n.m.r. spectra run in deuteriochloroform and deuterobenzene,<sup>104</sup> anticipating a shift difference between 4,4'-methoxyl signals and 3,3'-methoxyl signals, distinguishable according to the discussion of ellagic acid on page 136 .

It was planned to trideuteriomethylate<sup>8</sup> another sample of unknown A and by comparison of n.m.r. spectra in the same solvents locate the original methoxyl unambiguously.

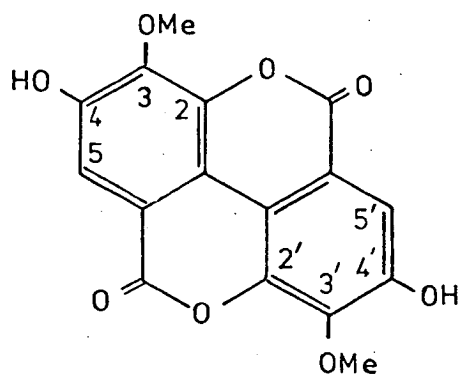
However the spectra of the methylated ether in deuteriochloroform and in deuterobenzene showed the same shift for all methoxyls, a reasonable inference despite the partial methylation. (Charts 42,43, pages 88,89).

Support for the identity of unknown A as 4-O-methylellagic acid rather than 3-O-methylellagic acid was obtained from analysis of its u.v. spectra.

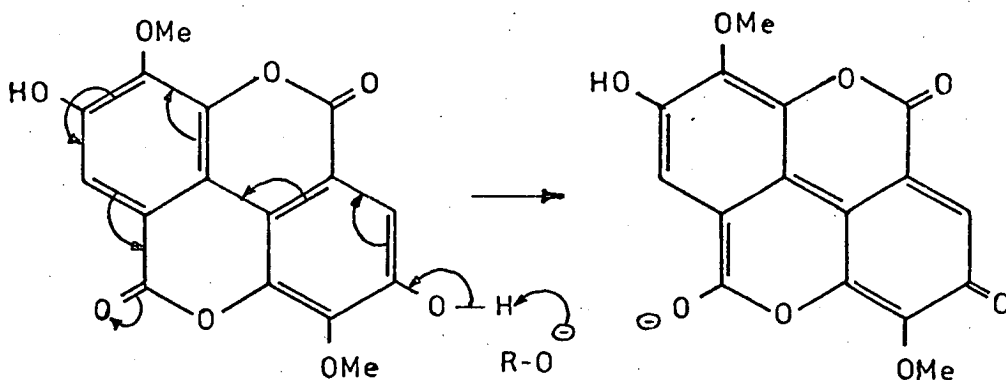
The comparison of the u.v. spectra of ellagic acid with those of its monomethyl ether in methanol alone, in methanol with fused sodium acetate added and in methanol with sodium methoxide added, is shown in Charts 44,45,46, pages 90,91,92.

Addition of fused sodium acetate to a methanolic solution of the monomethyl ether ionized the more acidic hydroxyls para- to the carbonyls<sup>35c</sup> to give a new band at 275 nm and a spectrum similar to that of ellagic acid after treatment with the same reagent.

(Chart 45, page 91).



( 24 )



( 25 )

( 26 )

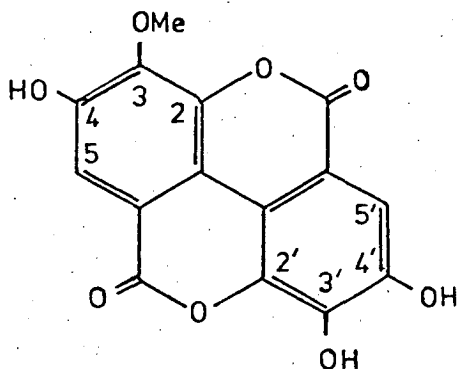
Other workers<sup>97</sup> have found no similar shift of the short wavelength band of the spectrum of the 3,3'-dimethylether (24). Of course both structures (22) and (23) of the monomethyl ether (page 153) have at least one ionizable hydroxyl para- to a carbonyl group so the evidence is inconclusive. But the size of the shift in the spectrum of the ether compares with the size of the shift in that of ellagic acid with two hydroxyls para- to carbonyls, suggesting free 3,3'-hydroxyls in the ether as in structure (22), the

4-O-methyl ether.

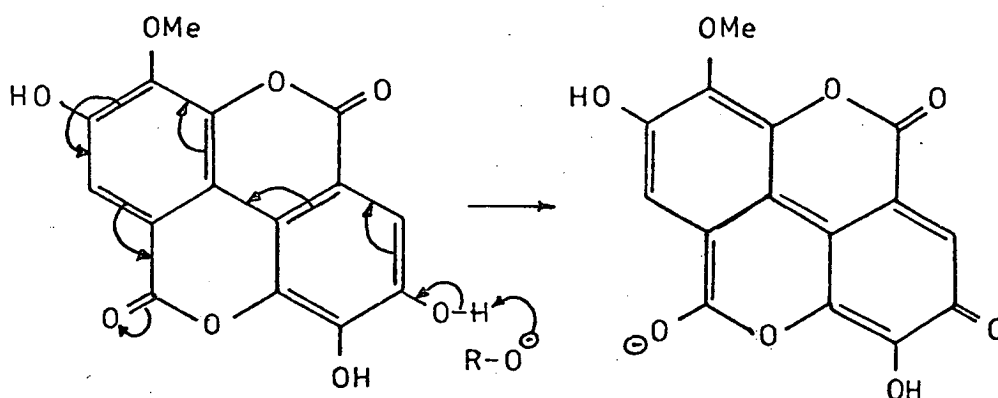
Further, in methanolic sodium methoxide solution, which is known to ionize all free hydroxyl groups,<sup>35c</sup> the spectra of ellagic acid and the ether were identical with the spectra of the compounds in acetate solution.

In contrast Scott<sup>97</sup> records that a sodium methoxide solution of the 3,3'-dimethyl ether (25) gives a distinctly different u.v. spectrum with an intense band at about 428 nm, tentatively assigned to transitions which result in a symmetrical chromophore system such as (25) to (26).

It seems reasonable that the ether structure (23), with a methyl group in the 3-position should also show this band at about 420 nm which would correspond to the analogous transition (27) to (28). But there is no evidence of such a band.



( 23 )



( 27 )

( 28 )

On the contrary the maxima of the spectrum are similar to those of the acetate spectrum for the 4,4'-dimethyl ether given by Scott.<sup>97</sup> This evidence supports the deduction that the methyl group in unknown A is at position -4 not position -3.

The above argument is summarised in Table 4 overleaf.

TABLE 4.

Absorption maxima (in nm) of ellagic acid derivatives.

COMPOUND	MeOH	MeOH-NaOAc	MeOH-NaOMe
Ellagic acid	256	255	255
	365	278	285
		360	
Unknown A	256	255	256
(4-O-methyl ellagic acid)	360	275	280
		358	
4,4'-dimethyl ellagic acid (Scott) <sup>97</sup>	253	256	256
	361	278	278
		361	361
3,3'-dimethyl ellagic acid (Scott) <sup>97</sup>	248	248	270
	372	373	320
			428

Finally, mass spectra and suggested fragmentation of ellagic acid and this monomethoxy derivative are given in Charts 16,47, pages 60,93.

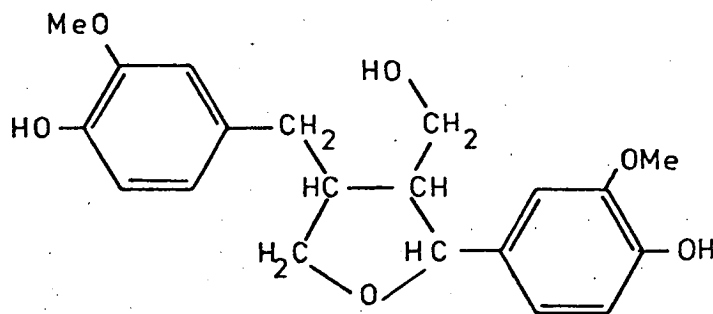
The combined u.v. and n.m.r. spectral evidence together with a satisfactory elemental analysis strongly supported the identification of Hillis' unknown A as 4-O-methylellagic acid, a compound hitherto unreported as a natural product.

4.1.4. UNKNOWN B (1,5-dimethyl-2,6-bis(trihydroxyphenyl) furo [ 1,5-c ] furan)

Hillis<sup>48</sup> suggested that the compound he termed unknown B should be provisionally identified as belonging to the ellagic acid or dilactone class because of its chromatographic behaviour.

From E. sieberi a compound has been isolated in this work with  $R_f$  values and u.v. colours comparable to those of unknown B but the spectral properties observed for this compound are those of lignans<sup>61</sup> rather than dilactones.

The u.v. spectrum of the E. sieberi compound with maxima about 215 nm and 280 nm, shown in Chart 48, page 94, resembled the published spectra<sup>61</sup> of the lariciresinol (29) class but was not comparable to the u.v. spectra of dilactones examined on preceding pages of this thesis.



Lariciresinol.

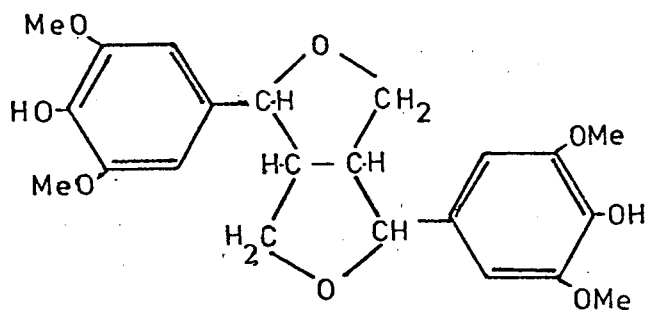
Other common classes of polyphenols with u.v. spectral maxima at ca. 290 nm are flavanones<sup>40,41</sup> and flavanonols<sup>40</sup> (3-hydroxyflavanones). However the isolated unknown B could not be reduced with sodium borohydride followed by exposure to acid vapour nor with magnesium in hydrochloric acid solution, so it was not a flavanone.<sup>39</sup> It could not be reduced with zinc and aqueous hydrochloric acid, so it was not a flavanonol.<sup>39</sup>

The mass spectrum of unknown B given schematically in Chart 49, page 95, showed a molecular ion of 390, far too large for flavonoids and greater than that of even the tetramethyl ether of ellagic acid (358) together with a fragmentation pattern quite different from that shown for the dilactone in Chart 16, page 60, supporting the u.v. evidence that unknown B is not an ellagic acid-like compound. The molecular ion of 390 could correspond to structure (30) shown in Chart 54, overleaf.

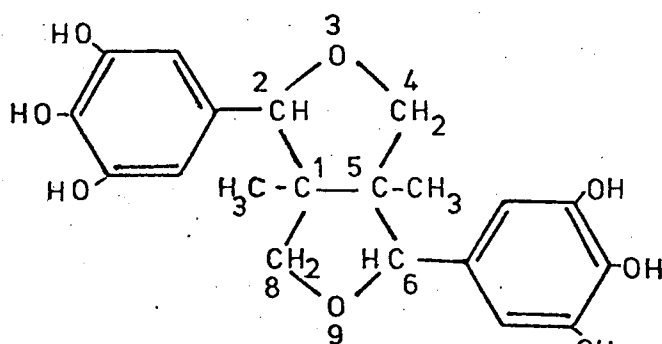
The isolated unknown B gave a positive red-purple colour with Mäule's test of chlorine fuming followed by sodium sulphite spray,<sup>80</sup> confirming the presence of the syringyl or pyrogallol residue (31) and gave a negative result with Pauly's reagent of diazotised sulphanilic acid<sup>85</sup> indicating the absence of a catechyl (32) or a guaiacyl (33) group, both results lending support for the proposed structure (30).



(34)

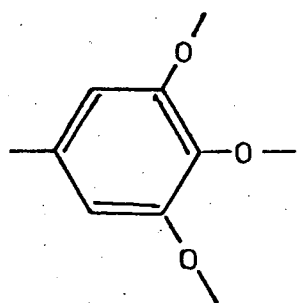
LIRIORESINOL

(30)

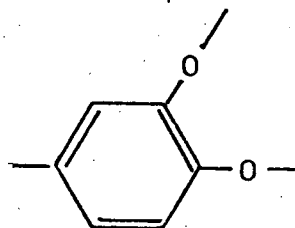
HILLIS' UNKNOWN B

( C H O = 390 )  
20 22 8

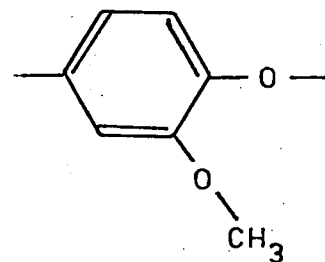
1,5-dimethyl-2,6-bis(3,4,5-trihydroxyphenyl)furo[1,5-c]furan



SYRINGYL  
(31)



CATECHYL  
(32)



GUAIACYL  
(33)

CHART 54.

The presence of hydroxyls in the syringyl group(s) was confirmed by the u.v. spectral bathochromic shifts with both aluminium chloride<sup>77</sup> and boric acid in acetate solution and by the blue colour on paper chromatograms when spots were sprayed with ferric chloride-potassium ferricyanide solution.

Strong hydroxyl absorption in both the inter-molecular and intra-molecular hydrogen bonding regions of the i.r. spectrum was in agreement with i.r. spectral data for lirioresinol (34) published by Pearl et al.<sup>86</sup>

The n.m.r. spectrum of unknown B showed no methoxyl signals but signals in both aliphatic and aromatic proton regions assignable to the protons in structure (30) represented three-dimensionally by diagram (35), (Chart 55, page 165 ). The spectrum was run in deuterodimethylsulphoxide with TMS as external lock, conditions which cause downfield shifts of signals relative to those observed with an internal lock. (Chart 50, page 96).

The singlets at  $\delta$  2.3 p.p.m. and  $\delta$  1.6 p.p.m., each integrating for three protons, have been tentatively assigned to aliphatic methyl groups in positions-1,5 in structure (30), the separate signals considered to be the result of the different environments experienced by the methyl groups near an aromatic nucleus or further from one respectively, as

represented in structure (35), page 165.

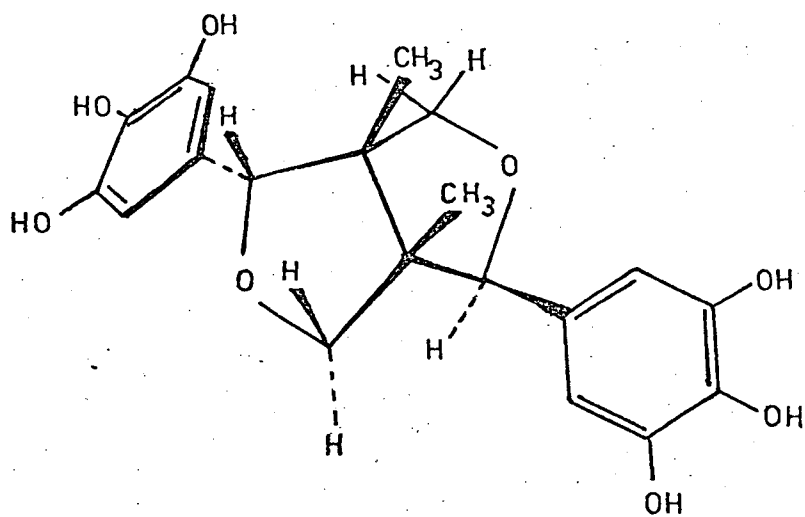
A structure such as (30) presents stereochemical problems, the first being the geometrical isomerism due to the bridging  $\beta$ -carbons. Molecular models showed the cis-form to be comparatively strain-free in contrast to the severely strained trans-form, supporting the conclusion of Dickey<sup>31</sup> that it is probable that such compounds exist only in the cis-form.

The lignan structure (30) suggested here for unknown B has two asymmetric centres with a possible two pairs of enantiomers. If we exclude the trans-isomers on the above grounds there is left one pair of possible isomers represented by configurations (36) and (37). (Chart 55, overleaf ).

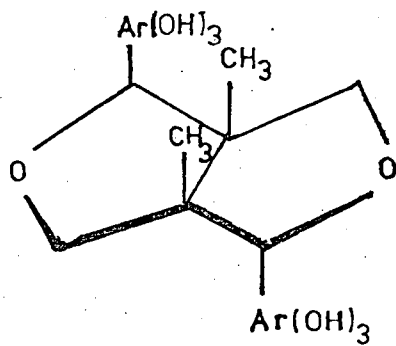
The n.m.r. spectral evidence discussed on page 163 seems to favour configuration (36) to explain the different environments for the aromatic protons and for the methyl groups in structure (30). Unknown B was dextro-rotatory.

The tentative conclusion of the present study is that unknown B is not a dilactone like ellagic acid as suggested by Hillis but is rather a lignan of the substituted furan type for which we suggest the provisional structure (30), 1,5-dimethyl-2,6-bis (3,4,5-trihydroxyphenyl) furo [ 1,5-c ] furan, a hitherto unreported lignan.

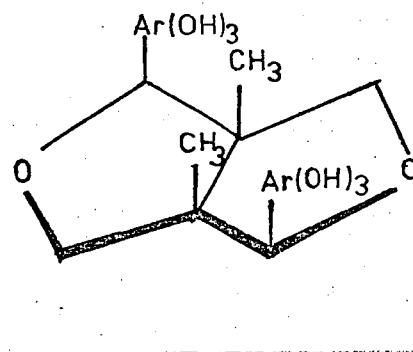
(35)



one enantiomer of cis-form of unknown B.



(36)



(37)

The d,l-pair of the cis-form of unknown B.

CHART 55.

4.1.5. UNKNOWN F (a tetrahydroxydibenzofuran-dicarboxylic acid isomer).

The compound isolated from E. sieberi with chromatographic behaviour similar to that of Hillis' unknown F<sup>48</sup> gave u.v. spectra with diagnostic reagents used throughout this work similar to the spectra of ellagic acid, suggesting that unknown F is a compound of similar chromophore system.

The n.m.r. spectrum showed no signals assignable to methoxyl protons expected for an ellagic acid derivative. The signals were a two-proton singlet at  $\delta$  7.94 p.p.m., downfield from the similar singlet in the spectrum of ellagic acid (Charts 51,17, pages 97,61) and broad multiplets in the region of phenolic and carboxylic hydroxyls integrating for six protons and removed by addition of deuterium oxide.

The i.r. spectrum (Chart 52, page 98 ) of unknown F was similar to that of ellagic acid with these differences: first, the strong band at ca.  $3350\text{ cm}^{-1}$  characteristic of the hydroxyl absorption in hydrogen bonded carboxylic acids,<sup>49</sup> with a broadening which suggested inter-molecularly bonded phenolic hydroxyls; secondly, a very strong band at ca.  $1710\text{ cm}^{-1}$ , at a frequency lower than that of the lactonic carbonyl of ellagic acid, seems attributable to the carbonyl of a carboxylic acid, undergoing hydrogen bonding.

The presence of one or more carboxyl groups

was supported by the liberation of carbon dioxide upon addition of the compound to saturated potassium bicarbonate solution while a positive reaction with ferric chloride indicated the presence of phenolic hydroxyls.

Structures (11) (ellagic acid) and (38), (39), (40), (41) and (42) were considered likely to have some spectral properties similar to those observed for unknown F. (Chart 56, overleaf).

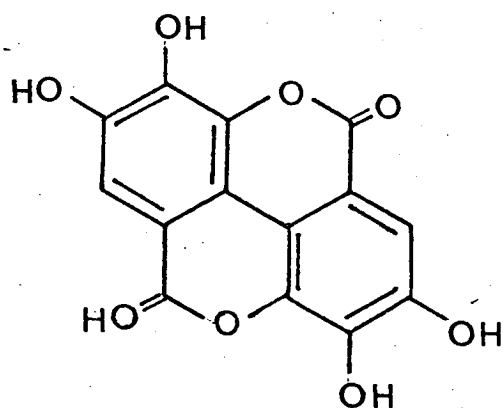
Structure (38) is flavellagic acid, a persistent impurity found by Perkin<sup>88,89</sup> in his synthesis of ellagic acid. When an authentic sample of flavellagic acid was mixed with a sample of authentic ellagic acid and a mixture of unknown F, unknown B and unknown A, Chart 57, page 169, was the result.

Distinct  $R_f$  values and chromatographic colours and a one-proton singlet in the n.m.r. spectrum made it certain that unknown F is not flavellagic acid.

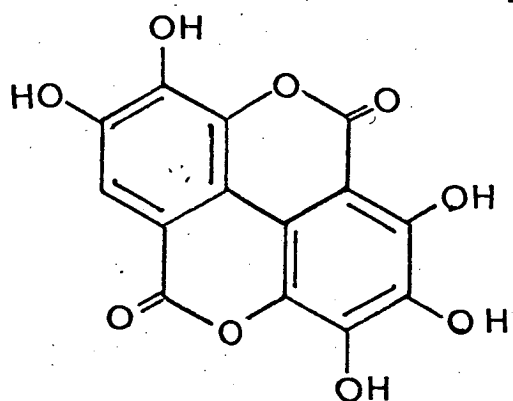
Structure (39) has a dioxo- group but the isolated unknown F gave a negative dioxo- test<sup>105</sup> and gave a molecular ion too small for this structure, which requires 336 mass units.

The remaining structures (40), (41) and (42), have structural features which seem to satisfy the requirements of unknown F.

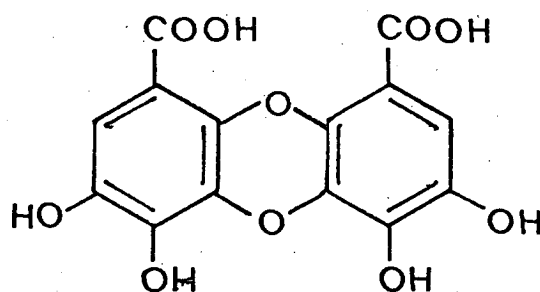
The presence of carboxyls and phenolic hydroxyls is supported by the bicarbonate and ferric chloride



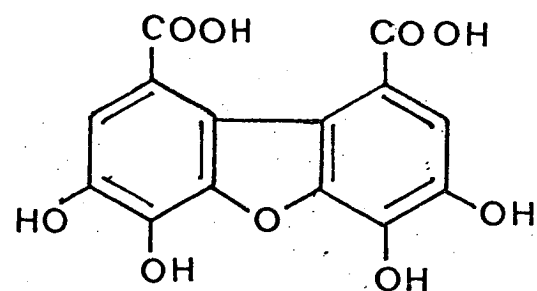
(11)



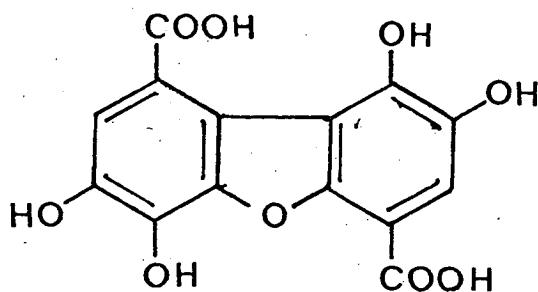
(38)



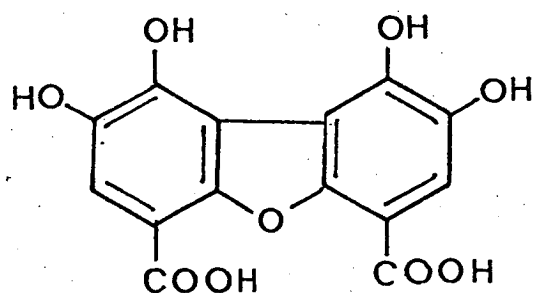
(39)



(40)



(41)



(42)

CHART 56. Possible structures of Unknown F.

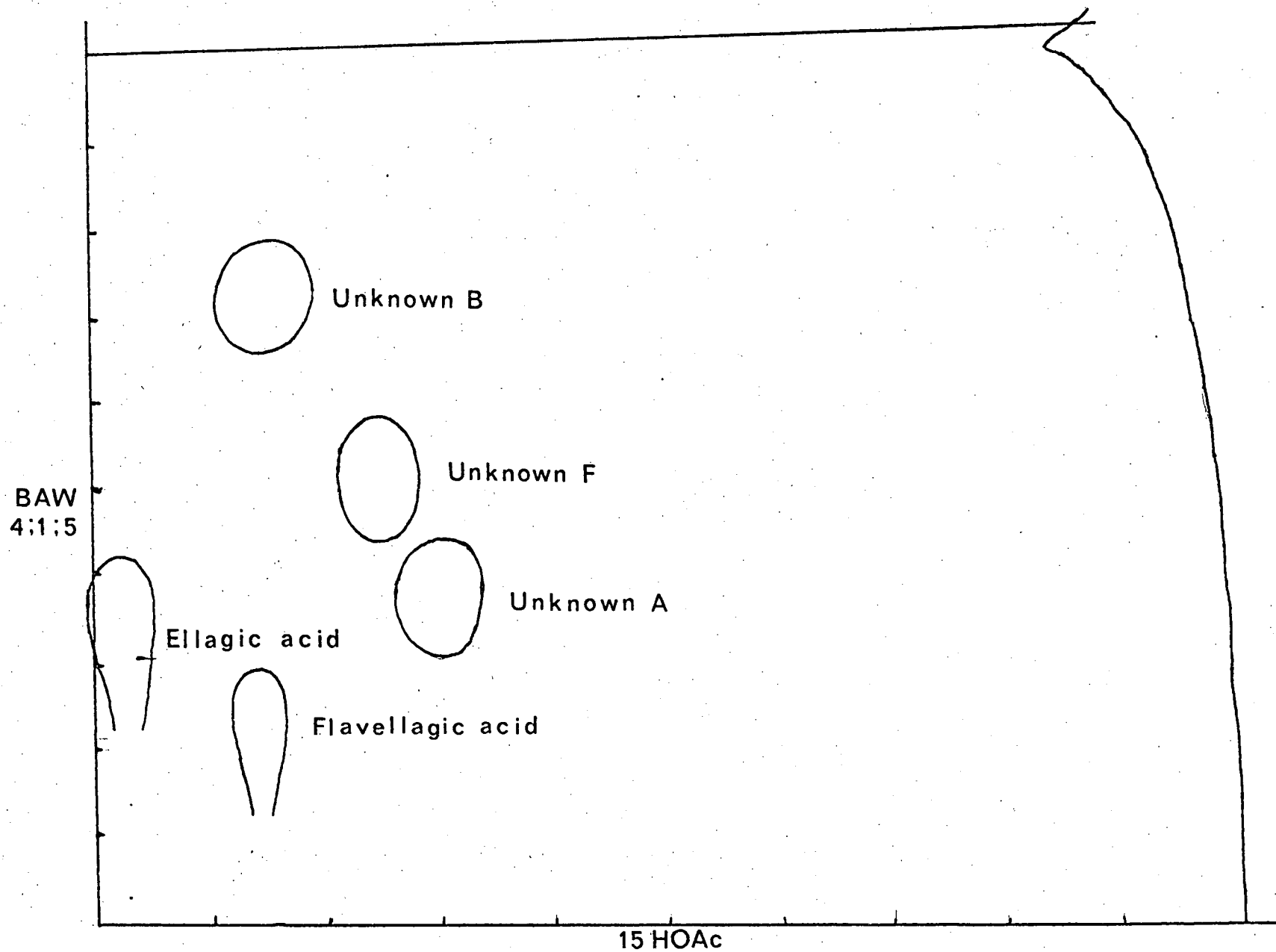


CHART 57. Chromatogram of Unknowns A,B,F and Ellagic, Flavellagic acids.



reactions respectively. The n.m.r. two-proton singlet indicates a chemically equivalent environment and a symmetrical molecule.

The downfield shift of this singlet relative to the similar singlet in the spectrum of ellagic acid can be explained by the greater deshielding afforded by the ortho- carboxyls in structures (40), (41) and (42).

Published u.v. data for dibenzofurans<sup>5</sup> showed clearly resolved bands at ca. 250 nm and ca. 300 nm, in substantial agreement with the spectrum of unknown F with bands at ca. 250 nm and ca. 305 nm. The ortho-dihydroxyls were confirmed by the u.v. shifts with aluminium chloride and boric acid in acetate solution.<sup>35a</sup>

Examination of molecular models of (40), (41) and (42) suggested that steric hindrance would be large between the carboxyls of (40), since they angle towards each other, less between the carboxyls in (42) which angle away from each other, and least in (41).

However further evidence is needed to distinguish between isomers (40), (41) and (42).

Finally the mass spectrum of unknown F showed a molecular ion of 320 (Chart 53, page 99), supporting the tentative identification of unknown F as a tetrahydrodibenzofuran-dicarboxylic acid.

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APPENDIX.

CHROMATOGRAPHIC BEHAVIOUR - SUMMARY.

1. Polyphenols of known structure.
2. Polyphenols of unknown structure  
(Hillis' unknowns).

1. Polyphenols of known structure.

E. DELEGATENSIS	E. SIEBERI	E. COCCIFERA	Polyphenol agylcones isolated in the present work.	COLOURS ON PAPER CHROMATOGRAMS						R <sub>f</sub> x 100 (Paper) in various solvents.		
				Day	365 nm	254 nm	a <sub>365</sub> NH <sub>3</sub>	pNA day	FeFe day	F	HA	BA/HA
+	-	-	gallic acid	-	m.	s.bu.	-	br.	bu.	65	35	58 42
+	-	-	gentisic acid	-	grey wh.	s.grey wh.	-	pk.	bu.	86	59	82 62
+	-	-	protocatechuic acid	-	m.	m.	s.m.	pk.	bu.	70	60	68 60
-	+	-	myricetin	p.y.	or.	-	b.y.	-	bu.	26	-	43 00
+	-	-	quercetin	p.y.	y.	y.	b.y.	-	bu.	40	-	64 00
-	-	+	kaempferol	p.y.	b.y.	b.y.	b.y.	-	bu.	55	-	82 00
+	-	-	ellagic acid	-	m.fl.	s.m.fl.	dull y.	-	bu.	33	-	34 02
+	-	-	(+)-catechin	-	m.	m.	s.m.	-	bu.	60	45	68 40

SOLVENTS: HA = 6% acetic acid.

BA/HA = butanol-acetic acid-water: 6:1:2;  
followed by 6% acetic acid.

F = Forestal: hydrochloric acid:  
acetic acid-water: 3:30:10.

COLOURS: b = bright; bu. = blue; fl. = fluorescent;  
gr. = green; l. = light; m. = mauve;  
or. = orange; pk. = pink; pu. = purple;  
s. = strong; wh. = white; y. = yellow.

SPRAYS: FeFe = 1% ferric chloride/1% potassium  
ferricyanide; pNA = diazotised  
p-nitroaniline.

2. Polyphenols of unknown structure.

E. DELEGATENSIS E. SIEBERI E. COCCIFERA	Polyphenol aglycones isolated in the present work.		COLOURS ON PAPER CHROMATOGRAMS						R <sub>f</sub> x 100 (Paper) in various solvents.		
			Day	365 nm	254 nm	a <sub>365</sub> NH <sub>3</sub>	pNA day	FeFe day	F	HA	BA/HA
- + -	11. unknown D	pinocembrin	-	pu.	pu.	l.y.	l.y.	bu.	93	05	92 07
- + -		alpinetin									
+ + -		0,0-dimethylpinocembrin									
- - +		apigenin									
- - +		sideroxylin									
- - +		4,5-dihydroxy-7-methoxy-6-methylflavone									
+ + -	5. unknown A	4-methoxyellagic acid	-	m.fl.	s.m.fl.	dull y.	-	bu.	48	-	54 02
+ + -	6. unknown B	1,5-dimethyl-2,6-bis(3,4,5-trihydroxyphenol) furo 1,5-c furan	-	m.fl.	s.m.fl.	dull y.	-	bu.	61	-	71 02
+ + -	13. unknown F	a dicarboxy-tetrahydroxy-dibenzofuran	-	s.l.y.	s.l.y.	fades	tan	bu.	-	15	47 12

SOLVENTS, SPRAYS, COLOURS as on the previous page.

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